AD_____

GRANT NO: DAMD17-94-J-4274

TITLE: Modulation of Molecular Markers by CLA

PRINCIPAL INVESTIGATOR(S): Doctor Henry J. Thompson

CONTRACTING ORGANIZATION: AMC Cancer Research Center
Denver, Colorado 80214

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual (14 Sep 94 - 13 Sep 95)

4. TITLE AND SUBTITLE

Modulation of Molecular Markers by CLA

5. FUNDING NUMBERS

DAMD17-94-J-4274

6. AUTHOR(S)

Doctor Henry J. Thompson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

AMC Cancer Research Center
Denver, Colorado 80214

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that has been shown to inhibit the development of experimentally-induced breast cancer. One reported explanation for this protective activity against carcinogenesis is that CLA is an antioxidant. Studies were completed during the current reporting period that indicate that CLA does reduce lipid peroxidation levels measured as malondialdehyde in mammary tissue, which is predominantly comprised of neutral lipid, but not in liver membrane phospholipid. CLA supplementation failed to alter tissue concentrations of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative DNA base damage, irrespective of its effect on tissue malondialdehyde levels. These observations were made under conditions of constitutive as well as induced oxidative stress. Collectively, these data indicate that the cancer inhibitory activity of CLA is unlikely to be due to its antioxidant activity. Given these observations we are now focusing our efforts on determining the effects of CLA on mammary gland development and the pathogenetically definable events leading to the genesis of breast cancer.

14. SUBJECT TERMS

Conjugated linoleic acid, mammary carcinogenesis, antioxidant activity, lipid peroxidation, malondialdehyde, oxidative DNA base damage, 8-hydroxydeoxyguanosine

15. NUMBER OF PAGES

58

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 9/15/95 Date

TABLE OF CONTENTS

Accesion For	
NTIS CRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

INTRODUCTION

PROPOSAL RELEVANCE. In 1981 Doll and Peto (1) estimated that nutrients and other dietary factors could account for a significant percentage of the risk for epithelial cancers in the United States and recently Doll (2) has suggested that approximately 35% of these cancers may be preventable via changes in dietary behaviors. Of the nutritional and dietary factors considered with regard to the risk for breast cancer, the role that the amount and type of dietary fat and calories play in the disease process has received prominent attention. This work has recently been reviewed (3,4). Two facts that have surfaced in this area of investigation are particularly relevant to the experiments being conducted. First, the level of caloric intake has a prominent effect on mammary tumorigenesis (3) and second, dietary fat has a specific effect on mammary tumorigenesis, but this effect is observed only when caloric intake is ad libitum (3). Our laboratory was one of the first to report the requirement for ad libitum intake for a fat specific effect on mammary tumorigenesis to be manifest (5), an observation that has recently been confirmed by others (6). It appears that this observation applies over a range of dietary fat concentrations. Given that a major health concern in the United States continues to be the consequences of intake of calories in excess of energy needs, it is probable that fat specific effects are being exerted in the U.S. population and other societies in which there is a surfeit of dietary calories.

As part of an overall public health initiative, Americans are being encouraged to eat less and exercise more in order to maintain "ideal" body weight, and to reduce the percent of dietary calories that they consume as fat (7). This advice is given with greatest specificity for prophylaxis of diseases of the heart, but these recommendations also apply to cancer, especially of the breast and colon. In general, it is recommended that dietary fat intake be reduced to $\leq$ 30% dietary calories with $\leq$ 10% provided as saturated fat, 10% as monounsaturated fat and 10% as polyunsaturated fat. An opportunity exists, therefore, to make recommendations about the specific fats that provide these calories. With regard to cancer, a principal interest lies in altering the type of polyunsaturated fatty acids (PUFA) that are being ingested. The question now receiving particular attention is whether all families of PUFA have similar effects on tumorigenesis and if individual fatty acids have selective effects on the mammary gland. ***The program of research being conducted on this grant specifically addresses this issue. We are investigating the cancer preventive activity of a specific fatty acid, conjugated linoleic acid (CLA), and we are studying various mechanisms that may account for its protective activity.***

CLA , a collective term that refers to conjugated dienoic derivatives of linoleic acid, is a naturally occurring substance in dairy products and in animal tissues. In a number of recent publications evidence has emerged indicating that CLA fed in the diet is a potent inhibitor of chemically-induced mammary carcinogenesis in the rat (8-11). This effect of CLA is in sharp contrast to that of linoleic acid which has been shown to stimulate the carcinogenic process in the same tumor model system in a dose dependent manner. Of added interest is the apparent potency of CLA in cancer prevention in comparison to other fatty acids reported to have cancer inhibitory activity. The most prominent among these are the fatty acids in fish oil. However, the amount of fish oil needed for cancer inhibitory activity usually exceeds 10% (w/w) in the diet. Recent work indicates that a level of CLA as low as 0.1% (w/w) was sufficient to produce a significant inhibition of mammary carcinogenesis. Thus, CLA is considerably more potent than any other fatty acid in inhibiting tumor development.

The potential relevance of these observations for cancer prevention in humans is considerable. In a direct extrapolation of the laboratory animal data to a 55 kg person, the amount of CLA required for cancer prevention would be equivalent to 2.8 g per day. The current estimate of CLA consumption per

day in the United States is 1 gram. The difference in these values is relatively small. Given that dietary levels of at least 1.5% CLA (w/w) can be fed chronically without adverse consequences, it appears that achieving a protective level of CLA consumption is quite feasible. CLA offers great potential as a preventive agent and could even be provided at effective levels via the food supply either via designer foods or as a dietary supplement.

In the work currently being conducted on this grant we are investigating the biological activity(s) of CLA that account for its cancer preventive activity. Our working hypothesis is that CLA affects the processes of clonal expansion and/or clonal selection via modulating genetic and/or epigenetic mechanisms obligatory for, or permissive to the carcinogenic process. This hypothesis is being evaluated by determining the effect of CLA on the expression of molecular markers relevant to the process of mammary carcinogenesis. These investigations may identify critical molecular events that can be targeted for cancer prevention.

References For This Section

1. Doll R. and Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J. Natl. Cancer Inst. 66:1191-1308.

2. Doll, R. (1992) The lessons of life: Keynote address to the Nutrition and Cancer Conference. Cancer Res. 52:2024s-2029s.

3. Welsch, C.W. (1992) Cancer Research (Suppl.) 52:2040S-2048S.

4. Welsch, C.W. (1992) In Exercise, Calories, Fat, and Cancer. Adv. in Exper. Med. Biol. 322, pp.203-222.

5. Thompson, H.J., Meeker, L.D., Tagliaferro, A.R. and Roberts, J.S. (1985) Nutr. Cancer 7:37-41.

6. Welsch, C.W., House, J.L., Herr, B.L., Eliasberg, S.J., and Welsch, M.A. (1990) J. Natl. Cancer Inst. 82:1615-1620.

7. National Research Council (1989) Diet and Health. Implications for reducing chronic disease risk. National Academy Press, Washington, D.C.

8. Ha, Y.L., Grimm, N.K. and Pariza, M.W. (1987) Carcinogenesis 8:1881-1887.

9. Pariza, M.W., and Yeong, L. HA. (1990) Med. Oncol. Tumor Pharmacother. 7:169-171.

10. Yeong, L Ha, Storkson, J. and Pariza, M.W. (1990) Cancer Res. 50:1097-1101.

11. Ip, C., Chin, S.F., Scimeca, J.A. and Pariza, M.W. (1991) Cancer Res. 51:6118-6124.

TECHNICAL OBJECTIVES OF THIS PROJECT

Objective 1. ***Does CLA inhibit the formation of oxidative damage to DNA?***

CLA has been reported to be a potent antioxidant in test tube assays, but its biological activity as an antioxidant is unclear. During the past year we investigated whether CLA alters oxidative damage to mammary gland DNA by measuring the accumulation of 8-hydroxydeoxyguanosine (8-OHG). Modification of this nucleoside has been implicated in site specific mutation of genes believed to affect the processes of clonal expansion and clonal selection. Our findings are reported below.

Objective 2. ***Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?***

While data published by our laboratory indicates that CLA suppresses proliferation of some mammary gland components during the course of normal development, it is unknown whether CLA has any effect on the expansion of colonies of cells that are transformed, and how such an effect is exerted. We have recently developed a model system in which this question can be evaluated. We will investigate whether CLA alters the process of clonal expansion of initiated mammary epithelial cells following carcinogen administration in vivo **during the next 12-month period**. This will be accomplished by measuring the time-related increase in mutant Ha-ras positive DNA using semi-quantitative PCR. If an effect is observed, we would determine whether the suppression of clonal expansion is due to a selective inhibition of cell proliferation and/or enhanced rate of cell loss using immunocytochemical and morphometric approaches.

Objective 3. ***Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?***

The hypothesis that forms the basis for this objective is that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. The key issue is to identify the genes that CLA modulates, and whether the effect is direct or indirect. CLA reduces but does not completely inhibit the occurrence of mammary carcinomas. We reason that treatment with CLA will result in the occurrence of a population of tumors that have a different spectrum of genetic defects than those tumors that occur in the absence of CLA treatment, i.e. that CLA will alter the process of clonal selection. Our approach will be to use this differential screening of tumors from rats fed control or CLA supplemented diets to identify candidate genes for further study. Preliminary data indicate that the mutant Ha-ras status of promoted tumors could be affected. Initial studies will be followed by investigations of the effects of CLA on the activities of these or other molecular markers of carcinogenesis as indicated by the data obtained, but at earlier time points in the disease process. Our goal is to determine how gene activity is affected in order to establish the causal basis for the cancer inhibitory activity of CLA.

BODY OF PROGRESS REPORT

The majority of effort during the first year of funding was directed to Technical Objective 1. The following sections detail the methods that were developed to meet the goals stated in this objective and the results obtained.

Materials and Methods

Source and composition of CLA and other dietary fats. The method of CLA synthesis from 99+% pure linoleic acid is detailed in reference (6, listed above). CLA was custom ordered from Nu-Chek, Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-,*t*9,*c*11- and *t*10,*c*12-CLA, constituted about 90% of the total. There were minimal variations in isomer distribution from batch to batch. Other fats used included: Mazola brand corn oil was obtained from Best Foods, Somerset, NJ, lard was purchased from Harlan Teklad, Madison, WI, menhaden oil was obtained from Marine Oil Test Program, U.S. Department of Interior, and palm oil was obtained from the Edible Oils Institute.

Animals and Diets

Animals. Female Sprague Dawley rats were used in the work reported. They were obtained from either Taconic Farms (Germantown, NY) or Charles River, Wilmington, Delaware. All rats were certified pathogen free.

Diet Formulations. A variety of diet formulations were used depending on the research question being addressed. All diets were modifications of the AIN-76A formulation and were designed to meet or exceed the known nutrient requirements of the rat unless otherwise specified.

Analytical Methods.

Analysis of urinary malondialdehyde (MDA). Following acid hydrolysis to release the bound form, MDA was derivatized with thiobarbituric acid (TBA) and the MDA-TBA adduct quantified by reverse phase HPLC with visible absorbance detection at 535nm. MDA content is expressed as nmol/mg creatinine.

In detail, 0.5 ml urine was combined with 5ul of an antioxidant solution containing 0.3M,2dp and 2% BHA in ethanol, and 40 ul concentrated HCL. The mixture was heated in a dry block at 96-99° for 4 and 3/4 hours. After samples had cooled slightly, 2 ml of TBA solution (1.11 % TBA in 74mM KOH) was added and the samples were heated at 96-99° for another 45 minutes. After cooling and immediately before HPLC analysis, samples were adjusted to a pH of 1.8 - 4.0 with 12N KOH. Previous method validation has confirmed that the presence of 2dp and BHA in urine samples during acid hydrolysis and TBA derivitzation prevents artifactual MDA contribution from food contamination in the urine, even with extreme contamination by menhaden oil containing diet. Creatinine was measured spectrophotometrically (Procedure 555, Sigma Diagnostics, St. Louis, MO 63178).

Determination of 8-OHdG and malondialdehyde in mammary tissue.

8-OHdG. For the assay of 8-OHdG, the various procedures of DNA purification from the mammary

gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and dG by HPLC, and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from our laboratory (11). Detector response was linear from 10 to >800 pg per injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues of 8-OHdG per 10^6 residues of dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Malondialdehyde (MDA). Tissue malondialdehyde was quantified as its thiobarbituric acid derivative with reverse phase HPLC and photometric absorbance detection at 535nm. In detail, mammary gland was homogenized with a Polytron in water containing 1% antioxidant solution (AOS: 0.3M dipyridyl and 2% BHA, in ethanol), 1 part mammary gland to 9 parts water (wt/vol). Homogenized samples were centrifuged at 6500 x g and fat plugs were removed, followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.25 mg protein was prepared for hydrolysis. The homogenate was combined, in glass tubes, with 7.5 ul AOS and enough water to bring the volume to 1.47 ml. 7.5 ul 5N HCl was added, and covered tubes were heated to 96° C for 3 hours. Tubes were cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. Tubes were centrifuged at 6500 x g for 10 min, and 1 ml of supernatant was then transferred from each to clean glass tubes. (The remaining supernatant and pellet were discarded.) 0.75 ml thiobarbituric acid (TBA) solution (1.11% TBA in 74 mM KOH) was added to each tube, and tubes were heated for 90 min for derivatization (to form TBA-MDA adduct). Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA-TBA adduct was separated using a 4.6 x 150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50mM potassium phosphate buffer, pH 6.0 delivered at 1.5 ml/min. Photometric absorbance detection was at 535nm. MDA was quantified by comparison of sample peak heights to those of standards, prepared from 1,1,3,3-tetramethoxypropane (TMP). To aliquots of stock standard were added water to 1.5 ml, 5 ul AOS, 1 ml TBA solution and 40 ul concentrated HCl. Standards were heated at 96° C for 14 min, cooled, and their pH adjusted to between 2.5 and 4.0 with 12N KOH. Final results were expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Determination of 8-OHdG concentration in liver DNA . The procedures described exhaustively herein contain significant changes from those previously described by us. The changes, such as eliminating phenol from the DNA isolation and adding BHT and 2-dp to buffers have been instrumental in reducing the contribution of artifacts to measured 8-OHdG. The importance of guarding against artifacts and their mistaken interpretation can not be over stated.

Isolation and enzymatic digestion of DNA from rat liver. DNA was isolated from liver with a phenol free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. In detail, 10ul of 26.4 mg/ml BHT was added to a 13 ml polypropylene screw cap tube, followed by 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulphate, pH 8.0, 400 ug/ml proteinase K (30 mAnson units/mg, cat # 24568, EC 3.4.21.14, from EM Science)) and approximately 75 mg frozen pulverized liver. The tube was inverted repeatedly to mix and incubated in a 50° water bath for 16-20 hrs, after which it was removed from the bath and allowed to cool briefly before adding 1 ml 7.5M ammonium acetate and mixing thoroughly. The resulting precipitate was removed from suspension by centrifugation at 19000g for ten minutes at 4°, and the supernatant decanted and extracted twice with 24:1 chloroform/isoamyl alcohol. Nucleic acids were precipitated by the addition

of 3 ml isopropanol, transferred to 1 ml silanized glass vials (Type I, Class A borosilicate glass, Waters Associates, Milford, MA) and the precipitate was washed with 70% EtOH before dissolution in 340 ul TE buffer (10mM Tris; 1mM EDTA; pH 8.0) containing 5mM dp. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% EtOH, dried briefly under reduced pressure without heat, and dissolved in100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides.

Chromatography of liver hydrolysate. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100Å) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was acheived on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported a residues 8-OHdG per million residues dG. The simultaneous analysis of both analytes from a single HPLC injection provided excellent precision without rigorously quantitative sample handling.

Results and Discussion

Appendix 1 contains one manuscript that is in press and another submitted to Carcinogenesis that provide a comprehensive perspective on the work conducted. What follows are relevant excerpts from those manuscripts, and data that is currently being prepared for publication.

Effect of CLA on the occurrence of lipid peroxidation and oxidative DNA damage in the mammary gland.

Results: In order to determine whether CLA would suppress oxidative damage in mammary tissue, the levels of malondialdehyde and 8-OHdG in mammary gland were analyzed. In this experiment, rats were fed the same corn oil or lard diet, with or without 1% CLA, as in the mammary carcinogenesis experiment shown in the following table (see manuscript 2, appendix 1 for additional information on this experiment).

Table 1. Mammary cancer prevention by CLA in rats fed either an unsaturated fat or a saturated fat diet[a]

Dietary fat	CLA	Tumor incidence	Total No. of tumors	% Inhibition[b]
corn oil	----	83.3%	68	
corn oil	1%	40.0%[c]	35[c]	49%
lard	---	80.0%	60	
lard	1%	40.0%[c]	32[c]	47%

[a] The unsaturated fat diet contained 20% corn oil, while the saturated fat diet contained 8% corn oil + 12% lard. There were 30 rats per group.

[b] Percent inhibition was calculated using the tumor number data.

[c] $P<0.05$ compared to the corresponding control group without CLA.

In the experiment reported in the following table, the animals were not treated with carcinogen. They were sacrificed after 2 months of feeding. Mammary tissue levels of malondialdehyde were significantly elevated in rats fed the corn oil versus the lard formulated diet ($p<0.001$), and the feeding of CLA was associated with lower levels of malondialdehyde ($p<0.001$). This effect was somewhat greater in mammary tissue from rats fed the greater amount of unsaturated dietary lipid (corn oil, 35% reduction; lard, 25% reduction, $p = 0.02$). Diet-associated differences in tissue levels of 8-OHdG were less remarkable. A 10-15% increase tissue 8-OHdG levels was associated with feeding the corn oil versus the lard diet ($p=0.08$); tissue levels of this oxidized base were unaffected by feeding CLA ($p=0.42$).

Table 2. Effect of CLA feeding on Malondialdehyde and 8-OHdG levels in mammary gland[a,b]

C L A Feeding

	No		Yes	
Dietary Fat	Malondialdehyde[c] nmol/mg Protein	8-OHdG[d] Residues/10^6dG	Malondialdehyde[c] nmol/mg Protein	8-OHdG[d] Residues/10^6dG
Lard	0.43 ± 0.03	3.38 ± 0.26	0.32 ± 0.02	3.75 ± 0.30
Corn Oil	1.39 ± 0.08	4.00 ± 0.26	0.90 ± 0.14	4.05 ± 0.20

[a] Rats were fed either the corn oil or lard diet, with or without 1% CLA for 2 months The fat diet contained either 20% corn oil or 12% lard + 8% corn oil.

[b] Results were expressed as mean $\pm$ SEM (n=9).

[c] By factorial analyses of variance, the following effects on malondialdehyde were noted. Type of fat: F-ratio 88.903, $p<0.001$; CLA: F-ratio 13.76, $p = 0.001$; Interaction between fat type and CLA: F-ratio 5.62, $p = 0.024$.

[d] By factorial analysis of variance, the following effects on 8-OHdG were noted:

Type of fat: F-ratio 3.18, p = 0.08; CLA: F-ratio 0.42, p = 0.42;
Interaction between fat type and CLA: F-ratio 0.37, p = 0.54.

Discussion of these data: The ability of CLA to suppress lipid peroxide was first described by Pariza's laboratory. In that work, linoleic acid was exposed to air and moderate heat, with or without a very small amount of CLA, for an extended period of time. Under those conditions, the degree of linoleic acid oxidation (peroxide value) was determined by the thiocyanate method. It was hypothesized that an oxidized derivative of CLA would act as the active antioxidant species rather than CLA itself. According to the proposed scheme, supported by spectrophotometric evidence, a β-hydroxy acrolein moiety would be introduced across the conjugated double bond of CLA following reaction with a hydroxyl or peroxyl radical and molecular oxygen. Antioxidant activity would result from chelation of iron by the β-hydroxy acrolein functional group, thereby interfering with the Fenton reaction.

The results presented above provides new clues relative to the effect of CLA on oxidative events *in vivo*. Tissue malondialdehyde levels were lower in mammary tissue of CLA treated rats, and the effect was somewhat greater in rats fed the more unsaturated dietary fat. The lipid evaluated is likely to represent neutral lipid contained in mammary gland adipocytes in which CLA was observed to be predominantly distributed (Table 2). This observation parallels the test tube assay data reported in. However, the measurement of 8-OHdG levels, which were affected by type of fat fed but not CLA, are likely to be a better indicator of whether CLA alters oxidative cellular events causally related to tumor promotion/progression. The lack of a measurable affect of CLA on 8-OHdG is consistent with the lack of significant accumulation of CLA in mammary epithelial cell phospholipid. This observation and the lack of effect of level of fat consumed on CLA-mediated inhibition of carcinogenesis argue against the likelihood that CLA inhibits mammary carcinogenesis by an antioxidant mechanism.

Effect of CLA on Lipid Peroxidation and DNA damage under conditons of oxidative stress.

Rationale. Having obtatined the above referenced results, we reasoned that CLA might still have an anitoxidant effect, and that our ability to detect this effect was limited by the experimental conditions studied.The role of oxidative stress in models in which CLA has been found to inhibit cancer is uncertain, making it difficult to determine the relevance of CLA's purported antioxidant properties. In the study reported here we sought to determine more definitively the scope and importance of CLA's antioxidant activity in vivo by looking for both systemic and tissue specific antioxidant effects of CLA in rats. Urinary MDA was used as a systemic index of lipid peroxidation, and tissue specific indices of oxidative damage (MDA and 8-OHdG) were assessed in liver under conditions in which evidence strongly suggests that oxidative stress can induce cancer. MDA was also measured in mammary gland, a tissue in which CLA has been shown to have potent cancer preventive activity.

Experimental diets were either of low peroxidation potential (Lpx) containing mostly palm oil (predominantly saturated fatty acids) and supplemented with adequate vitamin E, or of high peroxidation potential (Hpx) containing mostly menhaden oil (abundant polyunsaturated fatty acids) and deficient in vitamin E. Lpx and Hpx diets were each formulated both with and without CLA, with the expectation that if CLA does exert antioxidant activity, rats fed the CLA containing diets would have lower levels of MDA and 8-OHdG than those fed the respective non-CLA diets, and that the effect would be more pronounced with the Hpx diets.

Carcinogenic peroxisome proliferators are a novel class of hepatocarcinogens which do not directly

damage or interact with DNA and exhibit no direct genotoxicity, either by the parent compound or a metabolically activated species. Evidence that PP's induce carcinogenesis in rat liver via oxidative stress is extensive xx. Clofibrate (2- (p-chlorophenoxy) -2-methylpropionic acid ethyl ester) is a carcinogenic PP that is thought to cause oxidative stress in the livers of rats by greatly increasing peroxisomal beta-oxidation of fatty acids without an equivalent increase in catalase activity, thereby resulting in increased H_2O_2 accumulation and subsequent oxidatve attack of macromolecules by reactive oxygen species. The addition of clofibrate to all diets was designed to increase oxidative stress and provide greater opportunity for CLA to exert its putative antioxidant effects in a context were oxidative damage has been shown to be carcinogenic.

Oxidative DNA damage resulting in mutation is thought to be exacerbated by lipid peroxidation and may be the predominant mechanism by which oxidative stress ultimately causes or contributes to cancer.. If CLA's cancer inhibiting effects are due to antioxidant function, it is likely that oxidative DNA damage is effected. We measured 8-OHdG concentration, an indicator of such damage, in DNA extracted from liver, the target organ for clofibrate induced oxidative stress.

Results. Lipid peroxidation in mammary gland (Fig 1&2), as indicated by MDA content, was lower in the CLA fortified diet groups than in the respective non CLA diet groups, whereas liver MDA content (Fig. 3) was not affected by the addition of CLA to the diet. This evidence of antioxidant activity by CLA in mammary gland but not liver,is consistent withdata reported in table2, above. Our liver MDA data furthermore show no effect of CLA under conditions of high oxidative stress (PP's in diet) and when diets are of high peroxidative potential. Irrespective of the presence or absence of CLA in the diet, Hpx diet groups had elevated liver and mammary gland MDA content compaired to Lpx diet groups ($P \leq 0.001$) an observation that is consistent with expectation.

It is conceivable that lipid peroxidation in liver is indeed inhibited by CLA, but that rapid clearance precludes any observable difference in MDA accumulation. Such an effect would likely be reflected in urinary MDA excretion. However, our data suggest that this is not the case. Figure 4 summarizes the results of urinary MDA measurements. The presence of CLA in the diet shows no antioxidant activity as indicated by urinary MDA. There is in fact weak evidence that CLA has pro-oxidant characteristics when fed with clofibrate, particularly in the Hpx diet, but this trend was not statistically significant. The tendency for Hpx diet groups to show increased urinary MDA compaired to Lpx groups is consistent with expectation, but again the differences are not statistically significant. The impact of clofibrate as an oxidative stressor and the utility of urinary MDA measurement as an oxidative index are evidenced by the dramatic increase in MDA excretion observed when clofibrate was added to the diets.

The results of 8-OHdG measurements in DNA from liver, presented in Table 3, show no evidence for CLA a*cting as an antioxidant with respect to oxidative DNA damage. 8-OHdG average values among groups represent a narrow range and there were no statistically significant differences among groups. Considering the importance of maintaining genetic integrity and the inducible nature of DNA repair, it is not surprising that 8-OHdG measurements do not reflect the differences among groups seen in MDA measurements.

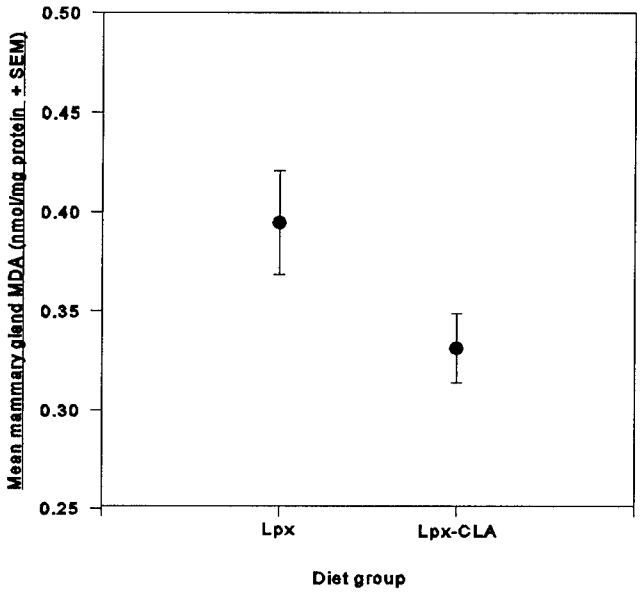

Fig 1. Effect of CLA in Lpx diets on mammary gland MDA. P = .063, two tailed t-test.

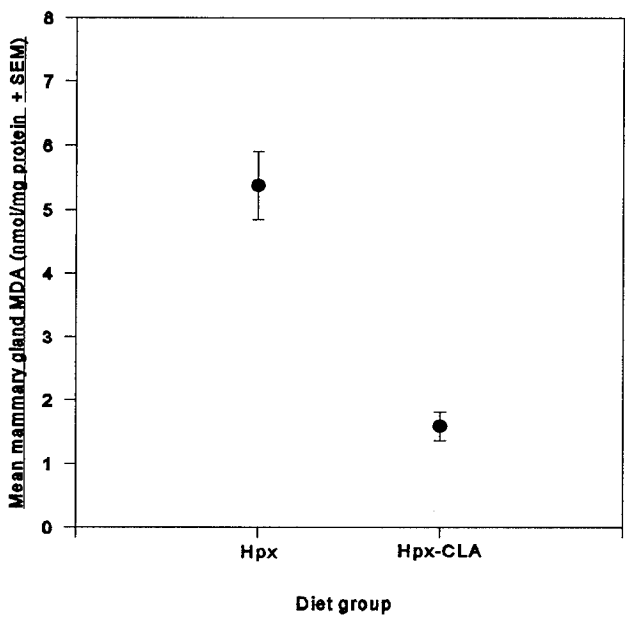

Fig. 2. Effect of CLA in Hpx diets on mammary gland MDA. P<.001.

Table 3. EFFECT OF CLA ON 8-OHdG LEVELS IN LIVER DNA

DIET	8-OHdG Residues/10^6 dG
Lpx	8.4 ± 1.7
Lpx/CLA	9.0 ± 1.3
Hpx	9.0 ± 1.2
Hpx/CLA	9.1 ± 2.3

Values are means ± SEM. The differences among groups were not statistically significant.

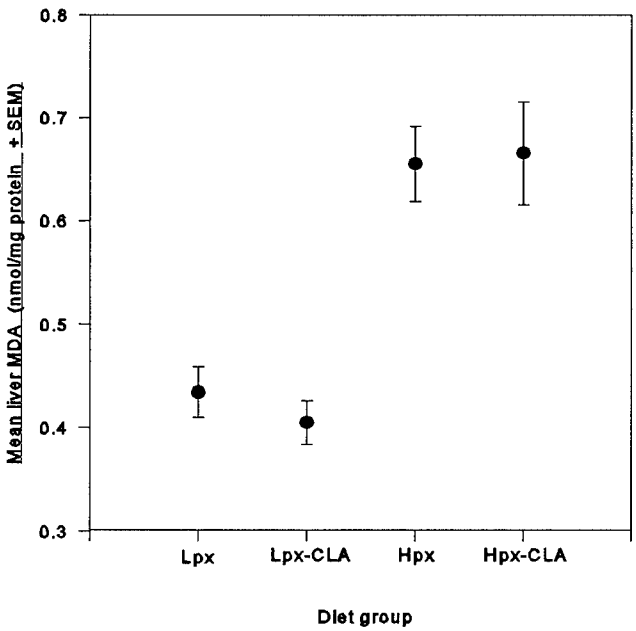

Fig 3. Effect of diet on liver MDA. n = 7-9 samples per group.

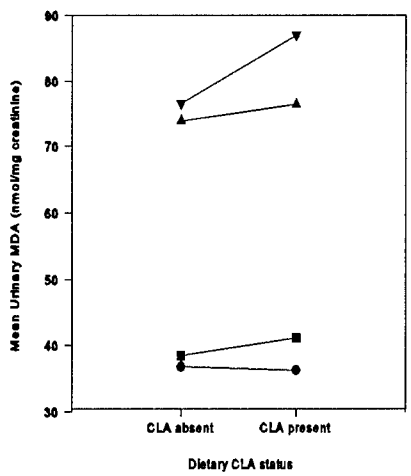

Fig. 4 Effect of dietary CLA on urinary MDA.
● = Lpx, ■ = Hpx, ▲ = Lpx-PP, ▼ = Hpx=PP, n=8-9 for each group.

Discussion. The evidence for antioxidant function by CLA only with respect to lipid peroxidation in mammary gland, and evidence that CLA is present in neutral lipid in greater proportions than in

phospholipid (in press) leads us to hypothesize that it is in neutral lipid that CLA exerts its antioxidant activity. If CLA were acting as an antioxidant by protecting membrane phospholipid, we would expect to find evidence in liver MDA measurements, where the phospholipid fraction represents a much greater proportion of total tissue fat than in mammary gland. That CLA's apparent antioxidant activity in mammary gland is not reflected in urinary MDA excretion may reflect less effective clearance of lipid peroxidation products from neutral lipid than from membrane phospholipid, where fatty acid integrety is critical and the reactive products of lipid peroxidation are likely to have more detrimental consequences. It is also conceivable that the observed differences in MDA abundance are the result of artifacts produced during processing. While BHT and 2dp are employed to minimize in vitro lipid peroxidation, and indeed in their absence MDA measurements are greater than 10 fold higher, the possibility of in vitro MDA production masquerading as in vivo lipid peroxidation can not be discounted entirely. If such is the case, CLA still exhibits significant antioxidant function, but that function may be biologically irrelevent. The confinement of CLA's antioxidant function to neutral lipid might also explain it's failure to inhibit 8-OHdG accumulation in DNA from liver or mammary gland.

References for this work

1. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989) Newly recognized anticarcinogenic fatty acids: Identification and quantification in natural and processed cheeses. *J. Agricul. Food Chem.*, **37**, 75-81.
2. Chin, S.F., Liu, W., Storkson, J.M., Ha, Y.L., and Pariza, M.W. (1992) Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *J. Food Comp. Anal.*, **5**, 185-197.
3. Ip, C., Carter, C.A., and Ip, M.M. (1985) Requirement of essential fatty acid for mammary tumorigenesis in the rat. *Cancer Res.*, **45**, 1997-2001.
4. Fischer, S.M., Conti, C.J., Locniskar, M., Belury, M.A., Maldve, R.E., Lee, M.L, Leyton, J., Slaga, T.J., and Bechtel, D.H. (1992) The effect of dietary fat on the rapid development of mammary tumors induced by 7,12-dimethylbenz(a)anthracene in SENCAR mice. *Cancer Res.*, **52**, 662-666.
5. Welsch, C.W. (1992) Relationship between dietary fat and experimental mammary tumorigenesis. A review and critique. *Cancer Res.*, **52**, 2040s-2048s.
6. Ip, C., Chin, S.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Res.*, **51**, 6118-6124.
7. Ip, C., Singh, M., Thompson, H.J., and Scimeca, J. (1994) Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Res.*, **54**, 1212-1215.
8. Ip, C., Scimeca, J.A., and Thompson, H. (1995) Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. *Nutr. Cancer*, In Press 1995.
9. Ha, Y.L., Storkson, J., and Pariza, M.W. (1990) Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.*, **50**, 1097-1101.
10. Park, J.-W. and Floyd, R.A. (1992) Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Radic. Biol. Med.* **12**, 245-250.
11. Haegele, A.D., Briggs, S.P., and Thompson, H.J. (1994) Antioxidant status and dietary lipid unsaturation modulate oxidative DNA damage. *Free Rad. Biol. Med.*, **16**, 111-115.
12. Horvath, P.M., and Ip, C.. (1983) Synergistic effect of vitamin E and selenium in the chemoprevention of mammary carcinogenesis in rats. *Cancer Res.*, **43**, 5335-5341.
13. Ip, C. (1990) Quantitative assessment of fat and calorie as risk factors in mammary carcinogenesis in an experimental model. *Prog. Clin. Biol. Res.*, **346**, 107-117.
14. Hahm, H.A. and Ip, M.M. (1990) Primary culture of normal rat mammary epithelial cells within a basement membrane matrix. I. Regulation of proliferation by hormones and growth factors. *In Vitro Cell Dev. Biol.*, **26**, 791-802.
15. Ip, C. (1987) Fat and essential fatty acid in mammary carcinogenesis. *Am. J. Clin. Nutr.*, **45**, 218-224.

16. Draper, H.H. and Hadley, M. (1990) Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology* **186**,421-431.
17. Cave, W.T., Jr. (1991) Dietary n-3 polyunsaturated fatty acid effects on animal tumorigenesis. *FASEB J.*, **5**, 2160-2166.
18. Ip, C., Ip, M.M., and Sylvester, P. (1986) Relevance of trans fatty acids and fish oil in animal tumorigenesis studies. *Prog. Clin. Biol. Res.*, **222**, 283-294.
19. Cohen, L.A., Chen-Backlund, J.-Y, Sepkovic, D.W., and Sugie, S. (1993) Effect of varying proportions of dietary menhaden and corn oil on experimental rat mammary tumor promotion. *Lipids*, **28**, 449-456.
20. Rose, D.P., Rayburn, J., Hatala, M.A., and Connolly, J.M. (1994) Effects of dietary fish oil on fatty acids and eicosanoids in metastasizing human breast cancer cells, *Nutr. Cancer*, **22**, 131-141.
21. Rose, D.P. and Connolly, J.M. (1993) Effects of dietary omega-3 fatty acids on human breast cancer growth and metastasis in nude mice. *J. Nat. Cancer Inst.*, **85**, 1743-1747.
22. Chin, S.F., Storkson, J.M., Liu, W., Albright, K.J., and Pariza, M.W. (1994) Conjugated linoleic acid (9,11- and 10,12-octadecadienoic acid) is produced in conventional but not germ-free rats fed linoleic acid. *J. Nutr.*, **124**, 694-701.
23. Buchanan, M.R., Haas, T.A., Legarde, M., and Guichardant, M. (1985) 13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J. Biol. Chem.*, **260**, 16056-16059.
24. Bull, A.W., Nigro, N.D., and Marnett, L.J. (1988) Structural requirements for stimulation of colonic cell proliferation by oxidized fatty acids. *Cancer Res.*, **48**, 1771-1776.
25. Baer, A.N., Costello, P.B., and Green, F.A. (1990) Free and esterified 13(R,S)-hydroxyoctadecadienoic acids: principal oxygenase products in psoriatic skin scales. *J. Lipid Res.*, **31**, 125-130.
26. Ramarathnam, N., Osawa, T., Namiki, M., and Kawakishi, S. (1988) Chemical studies on novel rice hull antioxidants. 1. Isolation, fractionation, and partial characterization. *J. Agric. Food Chem.*, **36**, 732-737.
27. Fraga, C., Shigenaga, M.K., Park, J.W., Degan, P., and Ames, B.N. (1990) Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA*, **87**, 4533-4537.
28. Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., and Nishimura, S. (1987) Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. *Nature*, **327**, 77-79.
29. Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) 8-Hydroxyguanine, an abundant form of DNA damage, causes G→T and A→C substitutions. *J. Biol. Chem.*, **267**, 167-172.
30. Darcy, K.M., Shoemaker, S.F., Lee, P.-P. H., Vaughan, M.M., Black, J.D., and Ip, M.M. (1995) Prolactin and epidermal growth factor regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture. *J. Cell Physiol.*, **163**, 346-364.
31. Darcy, K.M., Shoemaker, S.F., Lee, P.-P. H., Ganis, B.A. and Ip, M.M. (1995) Hydrocortisone and progesterone regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture. *J. Cell Physiol.*, **163**, 365-379.

CONCLUSIONS

Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that has been shown to inhibit the development of experimentally-induced breast cancer. One reported explanation for this protective activity against carcinogenesis is that CLA is an antioxidant. Studies were completed during the current reporting period that indicate CLA does reduce peroxidation levels measured as tissue malondialdehyde in neutral lipid rich tissues, but not in membrane phospholipid. Moreover, CLA supplementation failed to alter tissue concentrations of 8-hydroxydeoxyguanosine, an indicator of oxidative DNA base damage. These observations were made under conditions of constitutive as well as induced oxidative stress. Collectivelly, these data indicate that the cancer inhibitory activity of CLA is unlikely to be due to its antioxidant activity. This set of observations has permitted us to refocus our efforts on the effects of CLA on mammary gland development and the pathogenetically definable events leading to the development of breast cancer.

REFERENCES

The following is an overall list of references relevant to this research effort.

1. Doll R. and Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J. Natl. Cancer Inst. 66:1191-1308.

2. Doll, R. (1992) The lessons of life:Keynote address to the Nutrition and Cancer Conference. Cancer Res. 52:2024s-2029s.

3. Welsch, C.W. (1992) Cancer Research (Suppl.) 52:2040S-2048S.

4. Welsch, C.W. (1992) In Exercise, Calories, Fat, and Cancer. Adv. in Exper. Med. Biol. 322, pp.203-222.

5. Thompson, H.J., Meeker, L.D., Tagliaferro, A.R. and Roberts, J.S. (1985) Nutr. Cancer 7:37-41.

6. Welsch, C.W., House, J.L., Herr, B.L., Eliasberg, S.J., and Welsch, M.A. (1990) J. Natl. Cancer Inst. 82:1615-1620.

7. National Research Council (1989) Diet and Health. Implications for reducing chronic disease risk. National Academy Press, Washington, D.C.

8. Ha, Y.L., Grimm, N.K. and Pariza, M.W. (1987) Carcinogenesis 8:1881-1887.

9. Pariza, M.W., and Yeong, L. HA. (1990) Med. Oncol. Tumor Pharmacother. 7:169-171.

10. Yeong, L Ha, Storkson, J. and Pariza, M.W. (1990) Cancer Res. 50:1097-1101.

11. Ip, C., Chin, S.F., Scimeca, J.A. and Pariza, M.W. (1991) Cancer Res. 51:6118-6124.

12. Yuspa, S. H. and Harris, C.C. In Cancer Epidemiology and Prevention, (1982) D. Schottenfeld and J.F. Fraumeni (eds) W.B. Saunders, Philadelphia, pp. 23-42.

13. Cerutti, P. A. (1985) Science 227:375-381.

14. Harman, D.(1981) The aging process. Proc. Natl. Acad. Sci. USA 78:7124-7128.

15. Ames, B.N. Endogenous oxidative DNA damage, aging, and cancer. (1989) Free Rad. Res. Commun. 7:121-128.

16. Woo, J.W. and Floyd, R.A. (1992) Free Rad Biol Med 12:245-250.

17. Breimer, L.H. (1990) Molecular Carcinogenesis 3:188-197.

18. Malins, D.E. and Hobjectiveanot, R. (1991) Cancer Res 51:5430-5432.

19. Cheng, K.C. et al. (1992) J. Biol. Chem. 267:166-172.

20. Tchou, J. et al. (1991) Proc Natl Acad Sci 88:4690-4694.

21. Harris, C.C. (1991) Cancer Res (Suppl) 51:5023s-5044s.

22. Loeb, L. A. (1991) Cancer Res 51:3075-3079.

23. Patel, D., Singer, B. and Strauss, B.S. (1990) Cancer Res 50:2853-2856.

24. Kastan, M.B. et al. (1991) Cancer Res 52:6304-6311.

25. Matlashewski, G. et al (1986) Eur. J. Biochem. 154:665-672.

26. Moll, J.M. et al (1992) Proc Natl Acad Sci USA 89:7262-7266.

27. Nigro, J. M. et al (1989) Nature 342:705-708.

28. Miller, C.W. et al (1992) Cancer Res 52:1695-1698.

29. Suzuki, H. et al (1992) Cancer Res 52:734-736.

30. Bressac, B. et al (1991) Nature 350:429-431.

31. Hsu, I.C. et al (1991) Nature 350:427-428.

32. Coles, C. et al (1992) Cancer Res 52:5291-5298.

33. Nowell, P. C. (1976) Science, 194:23-28.

34a. Wainscoat, J.S. and Fey, M.F. (1990) Cancer Res. 50:1355-1360.

34b. Harris, C.C.(1991) Cancer Res. 51:5023s-5044s.

35. Steel, G.G. (1977) Growth kinetics of tumours. Clarendon Press, Oxford.

36. Weinberg, R.C. (1989) Cancer Res. 49:3713-3721.

37. Stanbridge, E.J. (1990) Annu. Rev. Genet. 24:615-657.

38. Boyd, J.A. and Barrett, J.C. (1990) Mol. Carcinog. 3:325-329.

39. Bouck, N. (1990) Cancer Cells 2:179-185.

40. Wyllie, A.H. (1987) J. Pathol. 153:313-316.

41. Walker, N.I., Bennett, R.E., and Kerr, J.F. (1989) Am. J. Anat. 185:19-32.

42. Thompson, H.J., Strange, R.S. and Schedin, P.S. (1992) Cancer Epidemiol. Biomarkers, Prev. 1:597-602.

43. Eldridge, S.R., Tilbury, L.F., Goldsworthy, T.L. and Butterworth, B.E. (1990) Carcinogenesis 11:2245-2251.

44. Kerr. J.F.R., Wyllie, A.H., and Currie, A.R. (1972) Br. J. Cancer 26:239-257.

45. Piacentin, M., et al. (1991) Eur. J. Cell Biol. 54:246-54, 1991; and Piacentini, M., et al. Cell Tissue Res. 263:227-235.

46. Barbacid, M. (1987) Annu. Rev. Biochem. 56:779-827.

47. Sukumar, S., Notario, V., Martin-Zanca, D., and Barbacid, M. (1983) Nature 306:658-661.

48. Zarbl, H. et al. (1985) Nature 315:382-385.

49. Mitra, G. et al. (1989) Proc. Nat. Acad. Sci. 86:8650-8654.

50. Lu, S. and Archer, M.C. (1992) Proc, Natl. Acad. Sci. 89:1001-1005.

51. Kumar, R., Sukumar, S. and Barbacid, M. (1990) Science 248:1101-1104.

52a.Zhang, R., Haag, J.D. and Gould, M.N. (1990) Cancer Res 50:4286-4290.

52b.Wang, B., Kennan, W.S., Yasukawa-Barnes, J., Lindstrom, M.J. and Gould, M.N. (1992) Cancer Res. 51:2642-2648.

53. Harris, J.R., Lippman, M.E., Veronesi, U. and Willet, W. (1992) N. Engl. J. Med. 327:473-480.

54. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and Press, M.F. (1989) Science 244:707-712.

55. Van de Vijver, M.J., Peterse, J.L. and Mooi, W.J., et al. (1988) N. Engl. J. Med. 319:1239-1245.

56. Mooi, W.J. and Peterse, J.L. (1992) Eur. J. Cancer 28:623-625.

57. Thompson, H.J. (1991) Carcinogenesis 12:2175-2179.

58. Thompson, H.J., Kennedy, K., Witt, M. and Juzefyk, J. (1991) Carcinogenesis 12:111-114.

59. Kumar, R., Sukumar, S. and Barbacid, M. (1990) Science 248:1101-1104.

60. Floyd, R.A.; Watson, J.J; Wong, P.K.; Altmiller, D.H.; Richard, R.C. (1986) Free Radical Res. Commun. 1:163-172.

61. Shigenaga, M.K.; Park, J-W.; Cundy, K.C.; Gimeno, C.J.; Ames, B.N. (1990) Methods in Enzymology 186:521-530.

62. Rickter, C.; Park, J-W.; Ames, B.N. (1988) Proc. Natl. Acad. Sci. USA 85: 6465-6467.

63a.Fraga, C.; Shigenaga, M.K.; Park, J.W.; Degan, P.; Ames, B.N. (1990) Proc. Natl. Acad. Sci. USA 87:4533-4537.

63b.Shibutani, S.; Takeshita, M.; Grollman, A.P. (1991) Nature 349:431-434.

64. Miller, C.W. (1992) Cancer Res. 52:1695-1698.

65. Piacentin, M. et al. (1991) Eur. J. Cell Biology 54:246-54 and Cell Tissue Res. 263:227-235.

66. Shibata, D. Hawes, D., Li, Z.H. et al. (1992) Am. J. Pathol. 141:539-543.

67. Barraclough, R. and Rudland, P.S. (1989) Eviron. Health Perspec. 80:39-48.

68. Russo, J. Gusterson, B.A. Russo, I. et al. (1990) Lab Invest. 62:244-275.

69. Talhouk, R.S., Streuli, C.H. Barcellos-Hoff, M.H. and Bissell, M.J. In: Fundamentals of Medical Cell Biology, vol. 2, Editor: E.E. Bittar. pp 137-178, (1991).

70. Strange, R.S., Feng, L., Saurer, S. and Friis, R. (1992) Development 115:49-58.

71. Harris, C.C. (1991) Cancer Res. 51:5023s-5044s.

72. Welsch, C.W. (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res. 45:3415-3443.

73. Gullino, P. , Pettigrew, H.M. and Grantham, F.H. (1975) Nitrosomethylurea as a mammary gland carcinogen in rats. J. Natl. Cancer Inst. 54:401-405.

74. Thompson, H.J. and Meeker, L.D. (1983) Induction of mammary gland carcinomas by the subcutaneous injection of MNU. Cancer Res. 43:1628-1629.

75. Young, S. and Hallowes, R.C. Tumors of the mammary gland In: V.S.Turusov(ed). Pathology of Tumors in Laboratory Animals. Vol 1, pp 31-74, Lyons, France:International Agency for Research on Cancer (1973).

76. Peto, R. (1974) Guidelines on the analysis of tumor rates and death rates in experimental animals. Br. J. Cancer 29:101-105.

77. Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, Ed. 6 Iowa University Press.

78. JK Beckman, T Yoshioka, SM Knobel, HL Green. Biphasic changes in phospholipid Hydroperoxide levels during renal ischemia/reperfusion. Free Radical Biology and Medicine, 11: 335-340, 1991.

79. T. Miyazawa, T. Suzuki, K. Fujimoto, and K.Yasuda. Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat. Journal of Lipid Research, 33: 1051-1059, 1992.

80. C. IP, SF Chin, JA Scimeca, and MW Pariza. Mammary Cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Research 51:6118-6124, 1991.

APPENDIX 1

Galley

Effect of Timing and Duration of Dietary Conjugated Linoleic Acid on Mammary Cancer Prevention

Clement Ip, Joseph A. Scimeca, and Henry Thompson

Abstract

Conjugated linoleic acid (CLA) is a minor fatty acid found predominantly in the form of triglycerides in beef and dairy products. Previous work by Ip and co-workers showed that free fatty acid-CLA at ≤1% in the diet is protective against mammary carcinogenesis in rats. The present study verified that the anticancer activities of free fatty acid-CLA and triglyceride-CLA are essentially identical. This is an important finding, because it rules out a nonspecific free fatty acid effect. In terms of practical implication, we can continue the in vivo *research with the less-expensive free fatty acid-CLA without compromising the physiological relevance of the data. A primary objective of this report was to investigate how the timing and duration of CLA feeding might affect the development of mammary carcinogenesis in the methylnitrosourea (MNU) model. We found that exposure to 1% CLA during the early postweaning and pubertal period only (from 21 to 42 days of age) was sufficient to reduce subsequent tumorigenesis induced by a single dose of MNU given at 56 days of age. This period incidentally corresponds to a time of active morphological development of the mammary gland to the mature state. In contrast to the above observation, a continuous intake of CLA was required for maximal inhibition of tumorigenesis when CLA feeding was started after MNU administration, suggesting that some active metabolite(s) of CLA might be involved in suppressing the process of neoplastic promotion/progression.*

(Nutr Cancer 24, 241–247, 1995)

Introduction

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of positional and geometric isomers of linoleic acid (1). The two double bonds in CLA are in Positions 9 and 11 or 10 and 12 along the carbon chain, thus giving rise to the designation of a conjugated diene. Each of the double bonds can be in the *cis (c)* or *trans (t)* configuration. CLA is normally found as a minor constituent in the lipid fraction of many different kinds of food (2). Meat from ruminants generally contains more CLA than meat from nonruminants. Cheese and other dairy products are also good sources of CLA, whereas seafoods and vegetable oils are not. Although the biochemistry of CLA has been documented for decades in the literature, little is known about its nutritional activity or requirement (3). More than 30 years ago, Bartlett and Chapman (4) first reported that CLA was an intermediate in the microbial biohydrogenation of linoleic acid

C. Ip is affiliated with the Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263. J. A. Scimeca is affiliated with the Nutrition Department, Kraft Foods, Inc., Glenview, IL 60025. H. Thompson is affiliated with the Laboratory of Nutrition Research, AMC Cancer Research Center, Denver, CO 80214.

in butter fat. Kepler and associates (5) subsequently discovered that a rumen bacterium, *Butyrivibrio fibrisolvens,* was able to convert linoleic acid to stearic acid via CLA. Microorganisms are not necessarily the major producer of CLA. Additional factors may facilitate the formation of CLA in cooked and processed foods. For example, grilling ground beef has been shown to increase the CLA content in beef fat by about fourfold (1). The new era of CLA and cancer prevention research began after the identification by Pariza's laboratory (6–8) of an antimutagenic and anticarcinogenic substance isolated in grilled ground beef.

In contrast to linoleic acid, which has been observed consistently to enhance mammary tumorigenesis in rodents over a wide concentration range (9–11), CLA expresses an inhibitory effect at ≤1% in the diet (12,13). The studies demonstrating a cancer-promoting effect of dietary linoleic acid were often conducted using vegetable oils (e.g., corn oil or safflower oil), which are rich in linoleate esterified to glycerol. CLA is likewise present naturally as a component of triglyceride in food. However, CLA was given as a free fatty acid in the previous animal mammary cancer prevention experiments (12,13). Triglyceride-CLA is not routinely used in vivo because of the prohibitive cost. However, the question has remained open as to whether the free fatty acid-CLA effect could be artifactual. One of the objectives of this study therefore was to compare the cancer-preventive efficacy of triglyceride-CLA with that of free fatty acid-CLA to evaluate the relevance of the latter form in biologic research.

Scanty information is available concerning the effect of interrupted vs. continuous CLA feeding on the benefit of cancer protection. As a first step in addressing this question, a major focus of the present report was to examine how the timing and duration of CLA feeding might affect the risk of mammary cancer development. All the experiments described below were carried out using the methylnitrosourea (MNU)-induced mammary tumor model in rats. MNU is a direct alkylating agent and does not require metabolic activation. Because the design involved CLA feeding immediately before or after carcinogen treatment, the MNU model is ideal for this purpose, inasmuch as it obviates any potential confounding influence of CLA on carcinogen metabolism.

Materials and Methods

Source of CLA

The method of CLA synthesis from >99% pure linoleic acid was detailed previously (12). The free fatty acid-CLA was custom ordered from Nu-Chek (Elysian, MN). Gas chromatographic analysis (12) showed the following composition: *c*9,*t*11- and *t*9,*c*11-CLA, 42.6%; *t*10,*c*12-CLA, 44.8%; *c*9,*c*11-CLA, 2.1%; *c*10,*c*12-CLA, 1.4%; *t*9,*t*11- and *t*10,*t*12-CLA, 2.8%; linoleic acid (unchanged parent compound), 1.8%; unidentified remainder, 4.5%.

A portion of this batch was set aside by Nu-Chek to prepare the triglyceride-CLA. The procedure involved reacting CLA methyl ester with triacetin in the presence of a sodium methoxide catalyst. Triglyceride-CLA was then separated from the mono- and diglycerides on silicic acid pads by repeated washings with petroleum ether-hexane. Because Nu-Chek has proprietary right to this procedure, no further information on the methodology can be released. High-pressure liquid chromatographic analysis of the triglyceride-CLA at the Kraft Foods Technology Center confirmed a purity of >99%. In addition, fatty acid analysis of the triglyceride-CLA indicated an isomer distribution nearly identical to that shown above, suggesting insignificant isomerization during the synthesis of the triglyceride form. CLA was the only fatty acid present in the synthetic triglyceride.

Design of Mammary Cancer Chemoprevention Experiments

Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in a room with a 12:12-hour light-dark cycle. Mam-

mary tumors were induced by a single injection of MNU (Ash Stevens, Detroit, MI; 50 mg/kg body wt ip). The MNU solution was prepared following the method of Thompson and Adlakha (14). Animals were palpated weekly to determine the time of appearance and location of tumors. At necropsy, the mammary glands were exposed for the detection of nonpalpable tumors. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by χ^2 analysis, and the total tumor yield between groups was compared by frequency distribution analysis, as described previously (15). Three mammary carcinogenesis experiments were carried out in accordance with the goals outlined in the **Introduction.**

The first experiment was designed to compare the efficacy of free fatty acid-CLA with that of triglyceride-CLA. Both forms of CLA were added to a modified basal AIN-76A diet (12) at a concentration of 1% by weight. The CLA-containing diets were fed from weaning until 56 days of age, when the animals were injected with MNU. CLA was removed at this point, and the rats were maintained on the basal AIN-76A diet until sacrifice (22 wks post-MNU). Control rats were fed the basal AIN-76A diet without CLA from weaning but were otherwise treated similarly.

The second experiment was designed to confirm that exposure to CLA during the period of active mammary gland development was critical for subsequent cancer protection. Animals were fed the 1% free fatty acid-CLA diet from weaning and were injected with MNU at 42 or 56 days of age. As in the above experiment, CLA was removed from the diet after MNU administration. The appropriate control rats were injected with MNU at either of the two time points but were fed the basal AIN-76A diet without CLA for the duration of the experiment.

The third experiment was designed to investigate the effect of different durations of CLA feeding after MNU treatment. Animals were injected with MNU at 56 days of age and were then given the 1% free fatty acid-CLA diet for one month, two months, or continuously until sacrifice (5 mos post-MNU).

Results

Table 1 summarizes the results of mammary cancer prevention by free fatty acid-CLA or triglyceride-CLA. In this experiment, the CLA diets were fed from weaning until 56 days of age (the time of MNU treatment), i.e., a duration of five weeks. Both forms of CLA were effective in tumor suppression. Furthermore the magnitude of the inhibitory effect was almost identical with the two reagents, suggesting that the free fatty acid form of CLA was absorbed as efficiently as the triglyceride form. This is an important observation, because it rules out a nonspecific free fatty acid effect. Additionally, it also means that the less-expensive free fatty acid-CLA can be used in animal feeding studies without compromising the interpretation of our findings.

The rat mammary gland undergoes marked morphological changes during the five-week

Table 1. Comparison of the Efficacy of Free Fatty Acid-CLA and Triglyceride-CLA in Mammary Cancer Prevention[a,b]

Dietary Treatment	Tumor Incidence	Total No. of Tumors
Control	24/30 (80.0%)	69
1% Triglyceride-CLA	16/30 (53.3%)*	37*
1% Free fatty acid-CLA	15/30 (50.0%)*	35*

a: Both forms of conjugated linoleic acid (CLA) were supplemented in the diet starting at weaning (21 days of age) and continuing until the time of methylnitrosourea administration at 56 days of age.
b: Statistical significance is as follows: *, $p < 0.05$ compared with corresponding control.

Table 2. Mammary Cancer Prevention by CLA Feeding From Weaning to the Time of MNU Administration[a–c]

Dietary Treatment	Age of MNU Administration, Days	Tumor Incidence	Total No. of Tumors
Control	42	25/30 (83.3%)	78
1% CLA	42	17/30 (56.7%)*	41*
Control	56	26/30 (86.7%)	81
1% CLA	56	16/30 (53.3%)*	47*

a: Animals were weaned at 21 days of age.
b: Free fatty acid-CLA was used in this experiment.
c: Statistical significance is as follows: *, $p < 0.05$ compared with corresponding control.

period after weaning (16). The above experiment suggests that exposure to CLA within this critical window of gland development is able to confer a lasting protective effect against mammary carcinogenesis in the absence of sustained treatment. Thompson and colleagues (17) recently showed that treatment with MNU as early as 28 days of age produced essentially the same carcinogenic response, as measured by tumor incidence and number. To verify a direct effect of CLA on the mammary gland, a second experiment was undertaken in which rats were injected with MNU at 42 or 56 days of age and the CLA diet was fed from weaning (21 days of age) to the time of MNU treatment. On the basis of the information from the first experiment, a 1% free fatty acid-CLA diet was used. Thus the length of CLA feeding was limited to three or five weeks, respectively, before MNU in the two supplemented groups. The results (Table 2) clearly indicate that exposure to CLA during the early postweaning and adolescent life span of the rat is sufficient in reducing the susceptibility of the mammary gland to subsequent carcinogen-induced neoplastic transformation. Rats injected with MNU at 42 days of age were maintained on the CLA diet for only three weeks, and yet the tumor-inhibitory activity of CLA with this protocol was very comparable to that observed with five weeks of CLA feeding (MNU injection at 56 days of age).

The last phase of the research was aimed at determining the effect of CLA feeding on the postinitiation phase of mammary carcinogenesis. CLA (1% as free fatty acid) was given immediately after MNU administration (dosed at 56 days of age) and was maintained for one month, two months, or continuously until termination of the experiment. The time course of tumor development in the different groups is shown in Figure 1. It is evident that short-term exposure to CLA for one or two months post-MNU was relatively ineffective in cancer protection. Significant inhibition ($p < 0.05$) was observed only in the group that received an uninterrupted supply of CLA in the diet.

Discussion

Although CLA has been shown to have a marked cancer-inhibitory activity (12,13), there is essentially no information about how it works or the conditions in which it is effective. In this regard, there are a number of noteworthy observations in the present study. First, the data in Table 1 indicate that the cancer-inhibitory activities of free fatty acid-CLA and triglyceride-CLA are very similar. This resolves the question of whether the previous reports of CLA's protective activity might be a nonspecific effect resulting from the feeding of CLA as a free fatty acid. The above observation also has practical value, in that it demonstrates the validity of continuing the future *in vivo* work with the more affordable free fatty acid-CLA without compromising interpretation of the data within the boundary of human relevance. Second, our experiments indicated that the timing of CLA feeding is clearly important in modulating mammary cancer risk. As demonstrated by the results summarized in Tables 1 and 2, exposure to CLA during the early postweaning and adolescent period is sufficient in

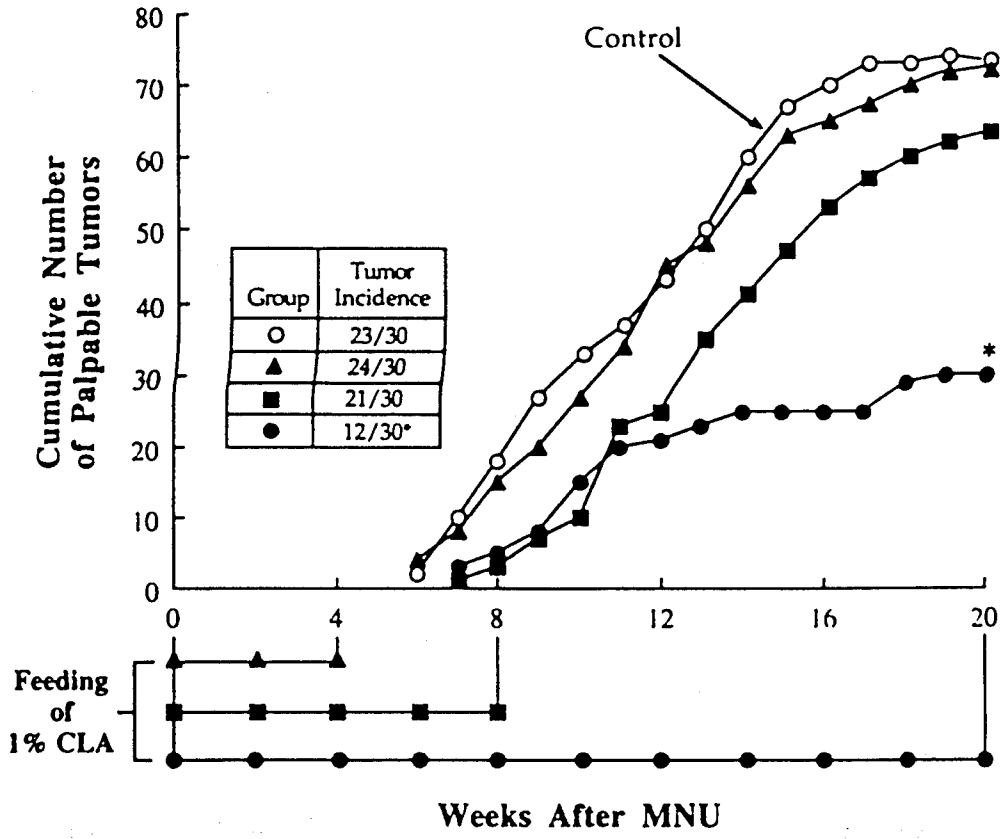

Figure 1. Effect of interrupted vs. continuous conjugated linoleic acid (CLA) feeding after methylnitrosourea (MNU) administration on mammary carcinogenesis. Duration of CLA feeding in 3 supplemented groups is indicated along *x*-axis time line by *filled symbols*, which match time course of mammary tumor development on main body of diagram. *Open circles*, control group without CLA supplementation. *, Statistically significant difference ($p < 0.05$) from control.

conferring some measurable degree of protection against subsequent chemically induced tumorigenesis in the mammary gland. This window of opportunity encompasses only a few weeks in the life span of the rat and yet appears to furnish certain epigenetic changes in rendering the target tissue less susceptible to neoplastic transformation. Third, it is evident from the experiment illustrated in Figure 1 that once the mammary cells have been initiated by a carcinogen, a continuous intake of CLA is required to achieve maximal inhibition of tumorigenesis.

According to the work of Russo and Russo (16), the rat mammary gland undergoes extensive morphological remodeling during the postnatal, prepubertal, and pubertal periods. At birth and in the first week of postnatal life, the mammary gland is made up of a single primary duct, which branches into several secondary ducts. These ducts end in dilated club-shaped structures called terminal end buds. During the second and third weeks, additional sprouting of the ducts occurs, leading to a sharp increase in the number of terminal end buds. After they reach a peak at weaning (21 days of age), the terminal end buds begin to reduce markedly in size and number because of their differentiation to alveolar buds and lobules. By about 40 days of age, these latter structures are far more prevalent than the terminal end buds and their population density is approaching a morphological state seen in the mature gland. We reported previously that CLA suppressed the level of bromodeoxyuridine labeling in the lobuloalveolar fraction (13). The determination was made at about 55–60 days of age after five weeks of CLA feeding, i.e., at a time when there were few terminal end buds left. Chemically induced

mammary carcinomas in the rat are believed to originate from terminal end bud cells and not from lobuloalveolar cells (16). Although it is reasonable to assume that the proliferative activity of the lobuloalveolar cells may be indicative of that of the progenitor terminal end bud cells, further work is clearly necessary to delineate the role of CLA in modulating the kinetics of mammary gland development. In view of our present observation that the feeding of CLA from weaning to 42 days of age (a duration of only 3 wks) is capable of protecting the mammary gland against tumorigenesis (Table 2), it is critical to find out whether this protective effect is due to 1) a general decrease in proliferative activity during gland morphogenesis, 2) a change in the time course of gland maturation, leading to a quantitative shift in gland composition, or 3) a specific response of target epithelial cells and/or the stromal component that surrounds the epithelial structures.

For a rational elucidation of how CLA might interfere with carcinogenesis after the mammary cells have been initiated, it might be profitable to consider the metabolic disposition of CLA *in vivo*. Being a fatty acid, CLA could potentially be 1) metabolized for energy, 2) incorporated as a component of membrane phospholipids and neutral lipids, or 3) converted to some other biologically active substances. The first route is unlikely to be of interest in the context of cancer prevention, whereas little is known about the significance of the latter two alternatives. We have unpublished data indicating that, in rats fed a 1% CLA diet, CLA is found in the phospholipid and neutral lipid fractions of the mammary epithelial cells. Our preliminary results also show that the incorporation of CLA as a percentage of total fatty acids is much higher in neutral lipids (~3%) than in phospholipids (~0.4%), suggesting that some selectivity might be involved in the compartmentalization of CLA into different classes of lipids. The presence of CLA in membrane phospholipids could conceivably be linked to the signal transduction pathway. This mechanism may modify the responsiveness to peptide stimulatory and/or inhibitory factors, which are known to play an important role in regulating the proliferation, morphogenesis, differentiation, and transformation of mammary epithelial cells. With respect to the neutral lipid CLA, it is possible that this pool may serve as the precursor to some as yet unidentified oxidized metabolites in the target tissue. Oxidation products of linoleic acid, including hydroperoxy-, hydroxy-, and oxooctadecadienoic acid, have been shown to express potent biologic activity in a number of different systems (18–20). It is possible that similar oxidation metabolites are produced from CLA. As illustrated by the data in Figure 1, the rate of tumor appearance rose after a short delay upon withdrawal of CLA feeding. This observation added weight to the idea of some active metabolites that are dependent on the availability of CLA. The intracellular effects of CLA might be multifocal. Our objective at this point is to suggest certain potentially fruitful areas of research that could contribute to the understanding of the mechanism of action of CLA in cancer prevention.

Acknowledgments and Notes

The authors thank Todd Parsons and Cathy Russin for technical assistance with the experiments. This work was supported by National Cancer Institute Grant CA-61763 from the National Institutes of Health (Bethesda, MD) and by a gift from Kraft Foods (Glenview, IL). Address reprint requests to Dr. Clement Ip, Dept. of Surgical Oncology, Elm & Carlton Sts, Buffalo, NY 14263.

Submitted 5 June 1995; accepted in final form 17 July 1995.

References

1. Ha, YL, Grimm, NK, and Pariza, MW: "Newly Recognized Anticarcinogenic Fatty Acids: Identification and Quantification in Natural and Processed Cheeses." *J Agric Food Chem* 37, 75–81, 1989.
2. Chin, SF, Liu, W, Storkson, JM, Ha, YL, and Pariza, MW: "Dietary Sources of Conjugated Dienoic Isomers of Linoleic Acid, a Newly Recognized Class of Anticarcinogens." *J Food Comp Anal* 5, 185–197, 1992.

3. Chin, F, Storkson, JM, Albright, KJ, Cook, ME, and Pariza, MW: "Conjugated Linoleic Acid Is a Growth Factor for Rats as Shown by Enhanced Weight Gain and Improved Feed Efficiency." *J Nutr* **124**, 2344–2349, 1994.
4. Bartlet, JC, and Chapman, DG: "Detection of Hydrogenated Fats in Butter Fat by Measurement of *Cis-Trans* Conjugated Unsaturation." *J Agric Food Chem* **9**, 50–53, 1961.
5. Kepler, CR, Hirons, KP, McNeill, JJ, and Tove, SB: "Intermediates and Products of the Biohydrogenation of Linoleic Acid by *Butyrivibrio fibrisolvens.*" *J Biol Chem* **241**, 1350–1354, 1966.
6. Pariza, MW, and Hargraves, WA: "A Beef-Derived Mutagenesis Modulator Inhibits Initiation of Mouse Epidermal Tumors by 7,12-Dimethylbenz[*a*]anthracene." *Carcinogenesis* **6**, 591–593, 1985.
7. Ha, YL, Grimm, NK, and Pariza, MW: "Anticarcinogens From Fried Ground Beef: Heat-Altered Derivatives of Linoleic Acid." *Carcinogenesis* **8**, 1881–1887, 1987.
8. Ha, YL, Storkson, J, and Pariza, MW: "Inhibition of Benzo[*a*]pyrene-Induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid." *Cancer Res* **50**, 1097–1101, 1990.
9. Ip, C, Carter, CA, and Ip, MM: "Requirement of Essential Fatty Acid for Mammary Tumorigenesis in the Rat." *Cancer Res* **45**, 1997–2001, 1985.
10. Fischer, SM, Conti, CJ, Locniskar, M, Belury, MA, Maldve, RE, et al.: "The Effect of Dietary Fat on the Rapid Development of Mammary Tumors Induced by 7,12-Dimethylbenz[*a*]anthracene in SENCAR Mice." *Cancer Res* **52**, 662–666, 1992.
11. Welsch, CW: "Relationship Between Dietary Fat and Experimental Mammary Tumorigenesis. A Review and Critique." *Cancer Res* **52**, 2040s–2048s, 1992.
12. Ip, C, Chin, SF, Scimeca, JA, and Pariza, MW: "Mammary Cancer Prevention by Conjugated Dienoic Derivative of Linoleic Acid." *Cancer Res* **51**, 6118–6124, 1991.
13. Ip, C, Singh, M, Thompson, HJ, and Scimeca, J: "Conjugated Linoleic Acid Suppresses Mammary Carcinogenesis and Proliferative Activity of the Mammary Gland in the Rat." *Cancer Res* **54**, 1212–1215, 1994.
14. Thompson, HJ, and Adlakha, H: "Dose-Responsive Induction of Mammary Gland Carcinomas by the Intraperitoneal Injection of 1-Methyl-1-Nitrosourea." *Cancer Res* **51**, 3411–3415, 1991.
15. Horvath, PM, and Ip, C: "Synergistic Effect of Vitamin E and Selenium in the Chemoprevention of Mammary Carcinogenesis in Rats." *Cancer Res* **43**, 5335–5341, 1983.
16. Russo, J, Tay, LK, and Russo, IH: "Differentiation of the Mammary Gland and Susceptibility to Carcinogenesis." *Breast Cancer Res Treat* **2**, 5–73, 1982.
17. Thompson, HJ, Adlakha, H, and Singh, M: "Effect of Carcinogen Dose and Age at Administration on Induction of Mammary Carcinogenesis by 1-Methyl-1-Nitrosourea." *Carcinogenesis* **13**, 1535–1539, 1992.
18. Buchanan, MR, Haas, TA, Lagarde, M, and Guichardant, M: "13-Hydroxyoctadecadienoic Acid Is the Vessel Wall Chemorepellant Factor, LOX." *J Biol Chem* **260**, 16056–16059, 1985.
19. Bull, AW, Nigro, ND, and Marnett, LJ: "Structural Requirements for Stimulation of Colonic Cell Proliferation by Oxidized Fatty Acids." *Cancer Res* **48**, 1771–1776, 1988.
20. Baer, AN, Costello, PB, and Green, FA: "Free and Esterified 13(R,S)-Hydroxyoctadecadienoic Acids: Principal Oxygenase Products in Psoriatic Skin Scales." *J Lipid Res* **31**, 125–130, 1990.

The Efficacy of Conjugated Linoleic Acid in Mammary Cancer Prevention is Independent of the Level or Type of Fat in the Diet

Clement Ip[1], Stephanie P. Briggs[2], Albert D. Haegele[2], Henry J. Thompson[2], Jayne Storkson[3] and Joseph A. Scimeca[4]

[1]Department of Surgical Oncology
Roswell Park Cancer Institute
Buffalo, NY 14263

[2]Division of Laboratory Research
AMC Cancer Research Center
Denver, CO 80214

[3]Food Research Institute
Department of Food Microbiology
University of Wisconsin - Madison
Madison, WI 53706

[4]Nutrition Department
Kraft Foods, Inc.
Glenview, IL 60025

Send all correspondence and proofs to:

Clement Ip, Ph.D.
Department of Surgical Oncology
Roswell Park Cancer Institute
Elm & Carlton Streets
Buffalo, NY 14263

Abbreviations

CLA,	conjugated linoleic acid
DMBA,	7,12-dimethylbenz[a]anthracene
8-OHdG,	8-hydroxydeoxyguanosine
MDA,	malondialdehyde
HPLC,	high pressure liquid chromatography
BHA,	butylated hydroxyanisole
TBA,	thiobarbituric acid

Abstract

The objective of the present study was to investigate whether the anticarcinogenic activity of conjugated linoleic acid (CLA) is affected by the composition of dietary fat consumed by the host. Because the anticancer agent of interest is a fatty acid, this approach may provide some insight into its mechanism of action depending on the outcome of these fat feeding experiments. Mammary cancer prevention by CLA was evaluated using the rat dimethylbenz[a]anthracene model. The results conclusively indicated that the magnitude of tumor inhibition by 1% CLA was not influenced by the level of fat (10-20% by weight of a blended fat) or the type of fat (corn oil versus lard) in the diet. It should be noted that these fat diets varied markedly in their content of linoleate. Fatty acid analysis showed that CLA was incorporated predominantly in mammary tissue neutral lipids, while the increase of CLA in mammary tissue phospholipids was minimal. Furthermore, there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in the phospholipid fraction. Collectively, these carcinogenesis and biochemical data suggest that the cancer preventive activity of CLA is unlikely to be mediated by interfering with the metabolic cascade involved in converting linoleic acid to eicosanoids. The hypothesis that CLA might act as an antioxidant was also examined. Treatment with CLA resulted in lower levels of mammary tissue malondialdehyde (an end product of lipid peroxidation), but failed to change the levels of 8-hydroxydeoxyguanosine (a marker of oxidatively damaged DNA). Thus while CLA may have some antioxidant function in vivo in suppressing lipid peroxidation, its anticarcinogenic activity cannot be accounted for by protecting the target cell DNA against oxidative damage. The finding that the inhibitory effect of CLA

maximized at 1% (regardless of the availability of linoleate in the diet) could conceivably point to a limiting step in the capacity to metabolize CLA to some active product(s) which is essential for cancer prevention.

Introduction

Conjugated linoleic acid (CLA) is a positional and geometric isomer of linoleic acid (1). It is a minor fatty acid found preferentially in beef and dairy products (2). In contrast to linoleic acid which has been found consistently to enhance mammary tumorigenesis in rodents over a wide concentration range (3-5), CLA expresses an inhibitory effect at levels of 1% or less in the diet (6,7). Recently, we have described two distinct activities of CLA in mammary cancer prevention with the use of the methylnitrosourea (MNU) model (8). First, exposure to CLA during the early post-weaning and peri-pubertal period only (from 21 to 42 days of age) is sufficient to block subsequent tumorigenesis induced by a single dose of MNU given at 56 days of age. This observation suggests that CLA is able to effect certain changes in the immature mammary gland and render it less susceptible to neoplastic transformation later in life. Second, CLA is also active in suppressing tumor promotion/progression. However, the mode of action is different from the first in that once the mammary cells have been initiated by a carcinogen, a continuous intake of CLA is necessary to achieve maximal inhibition.

The above cited studies on CLA chemoprevention (6-8) were carried out in rats fed a 5% (w/w) fat diet formulated with corn oil. Currently, there is no information as to whether an increase in the level of fat or a substitution in the type of fat in the diet might affect the cancer inhibitory efficacy of CLA. The experiments described in this paper were designed to address this question. Because the anticancer agent of interest is a fatty acid, it is anticipated that the approach will provide some insight into its mechanism of action depending on the outcome of these fat feeding experiments. For the fat level experiment, a custom-formulated fat blend was

used that simulates the fatty acid composition of the US diet. For the fat type experiment, a 20% (w/w) fat diet containing either corn oil (exclusively) or lard (predominantly) was used. The choice of these fat diets is explained in greater detail in the Methods section. Mammary cancer prevention by CLA under these various dietary conditions was evaluated using the rat dimethylbenz[a]anthracene (DMBA) model.

Previous work by Ha et al (9) suggested that CLA is a potent antioxidant. At a molar ratio of 1 part CLA to 1,000 parts linoleic acid, peroxide formation was reduced by more than 90% in a test-tube assay. In fact, CLA was superior to tocopherol in this regard. In order to investigate whether interference with oxidative processes in cells might be implicated in cancer prevention by CLA, we examined the effect of CLA on two markers of cellular oxidative damage in the mammary tissue of rats fed either a high-corn oil (unsaturated fat) or high-lard (saturated fat) diet. These markers were malondialdehyde, an end product of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), an oxidized base isolated from DNA. Lipid peroxidation products have been implicated in mediating the formation of 8-OHdG in DNA (10). A recent publication from Thompson's laboratory has also reported that the number of 8-OHdG residues in mammary gland DNA increased in proportion to the degree of fatty acid unsaturation (as determined by iodine value) in the diet oils (11). More importantly, the rate of increase was sensitive to the presence or absence of nutritional levels of antioxidants such as vitamin E and selenium. Because of the above findings, we felt that these markers would be appropriate in assessing whether the antioxidant activity of CLA is manifest *in vivo*. Our goal was to investigate

the possible relationship between the modulation of oxidative damage and the efficacy of cancer protection by CLA.

Materials and Methods

Source and composition of CLA and other dietary fats

The method of CLA synthesis from 99+% pure linoleic acid was detailed in our earlier publication (6). CLA was custom ordered from Nu-Chek, Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-,*t*9,*c*11- and *t*10,*c*12-CLA, constituted about 90% of the total. From our experience over several years, we have found that there were minimal variations in isomer distribution from batch to batch.

A "vegetable fat blend" was prepared by Kraft Foods, Inc., at their Technology Center in Glenview, IL. This fat blend was designed specifically to simulate the fatty acid composition in the average American diet. It consisted of 39.5% soybean oil, 22% palm oil, 12.5% high oleic-sunflower oil, 9% cottonseed oil, 8.5% coconut oil and 8.5% cocoa butter. The reason that plant oils were used exclusively was to minimize the CLA content of the fat blend. Gas chromatographic analysis showed the following composition: C8:0, 0.9%; C10:0, 0.7%; C12:0, 5.1%; C14:0, 2.3%; C16:0, 18.8%; C16:1, 0.2%; C18:0, 5.6%; C18:1, 31.8%; C18:2, 29.5%; C18:3, 3.4%; C20:0, 0.4%; C22:0, 0.3%; and CLA, not detectable. The above "vegetable fat blend" has a polyunsaturate/monounsaturate/saturate fatty acid ratio of 1/1/1, which is identical to that found in the typical US diet.

Two other commercial fats were used in this study: Mazola brand corn oil was obtained from Best Foods, Somerset, NJ, and lard was purchased from Harlan Teklad, Madison, WI. Lard contains about 0.3 mg CLA per g of fat.

Design of mammary cancer chemoprevention experiments

Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in an environmentally controlled room with a 12-h light/12-h dark cycle. Mammary tumors were induced by a single i.g. dose of 7.5 mg DMBA at 50 days of age. Animals were palpated weekly to determine the time of appearance and location of tumors. At necropsy, the mammary glands were exposed for the detection of nonpalpable tumors. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by chi-square analysis, and the total tumor yield between groups was compared by frequency distribution analysis as described previously (12).

The first experiment involved feeding rats a diet containing different levels of the "vegetable fat blend" at 10%, 13.3%, 16.7% and 20% by weight, with or without 1% CLA. Thus there were a total of 8 dietary treatment groups in this design. All diets, which were prepared according to the AIN-76 formulation (6), were started 1 week before DMBA and continued until sacrifice (23 weeks post DMBA). Ip has previously described the method of nutrient adjustment for diets containing different levels of fat so that the intake of protein, vitamins, minerals and calories was similar among the different groups (13).

At necropsy, the uninvolved (non-tumor bearing) mammary glands from selected groups were excised and immediately dropped in liquid nitrogen. Upon removal from storage at -80°C, the frozen samples were pulverized and total fat was extracted by chloroform/methanol. The separation of phospholipids and neutral lipids was achieved with the use of a Sep-Pak silica cartridge as described in our earlier publication (6). Gas chromatographic analysis of the fatty

acid methyl esters (including CLA) was determined by the method reported previously by Chin et al (2).

The second experiment involved feeding a diet containing either 20% corn oil or a mixture of 8% corn oil + 12% lard, both with or without 1% CLA. Lard was chosen over tallow because of the much lower CLA content in lard (4 mg CLA/g of fat in tallow versus 0.3 mg CLA/g of fat in lard). The 12% lard in the diet therefore contributed <4 mg of CLA per 100 g diet, an amount that was insignificant compared to the level of 1% CLA used in this experiment. It should be noted that the lard diet also contained 8% corn oil. The reason was based on the previous finding of the high linoleate requirement for mammary tumorigenesis in the DMBA model (3,14). Similar to the above protocol, the feeding of the corn oil or lard diet $\pm$ 1% CLA was started 1 week before DMBA and continued until sacrifice.

The third experiment involved feeding a 20% corn oil diet with either 0.5%, 1% or 1.5% CLA. The purpose was to determine the dose response to CLA in the presence of a linoleate-rich diet, and to compare the results obtained here with our previous study of CLA efficacy (also at 0.5%, 1% or 1.5%) in rats fed a 5% corn oil diet (6). Corn oil consists of about 60% linoleate. Thus the 5% and 20% corn oil diets contain about 3 g and 12 g of linoleate, respectively, per 100 g of diet.

Determination of malondialdehyde and 8-OHdG in mammary tissue

A separate experiment was set up to evaluate the effect of CLA on markers of lipid peroxidation and cellular oxidative damage in the mammary gland. Rats were fed the same corn oil or lard diet, with or without 1% CLA, as described in the above section. However, they were

not treated with DMBA and the feeding period only lasted for 2 months. At necropsy, the abdominal inguinal mammary gland chains (glands 4-6) were excised and dropped immediately in liquid nitrogen.

Tissue malondialdehyde (MDA) was quantified as its thiobarbituric acid derivative with reverse phase HPLC and photometric absorbance detection at 535nm based on an extensive modification of the method described by Draper and Hadley (15). Mammary gland samples were homogenized with a Polytron in water containing a 1% antioxidant solution (AOS: 0.3M dipyridyl and 2% BHA, in ethanol), 1 part mammary tissue to 9 parts water (wt/vol). The samples were centrifuged at 6500 x g and the fat plugs were removed, followed by further homogenization to re-suspend the pellet. Since optimal reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.2 mg protein was prepared for hydrolysis. The homogenate was combined with 7.5 µl 5N HCl, 7.5 µl AOS and enough water to bring the volume to 1.5 ml. The covered tubes were heated to 96° C for 3 hours. They were cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. After centrifugation at 6500 x g for 10 min, 1 ml of supernatant was then transferred to a clean glass tube. An aliquot of 0.75 ml thiobarbituric acid (TBA) solution (1.11% TBA in 74 mM KOH) was added to each tube, followed by heating for 90 min to form the MDA-TBA adduct. Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA-TBA adduct was separated using a 4.6 x 150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50mM potassium phosphate buffer, pH 6.0, delivered at 1.5 ml/min. Photometric absorbance detection

was at 535nm. Malondialdehyde was quantified by comparison of sample peak heights to those of the standard prepared from 1,1,3,3-tetramethoxypropane . The final results are expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and dG by HPLC, and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from Thompson's laboratory (11). The only modification introduced here was the elimination of phenol from the DNA isolation procedure. Detector response was linear from 10 to >800 pg per injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues of 8-OHdG per 10^6 residues of dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Results

Table 1 summarizes the mammary cancer chemoprevention data of CLA in rats fed different levels of fat. Tumor incidence at the time of necropsy was significantly reduced ($P<0.05$) by CLA treatment in each of the four fat groups. In the absence of CLA supplementation, the total number of tumors increased by approximately 40% (from 71 to 98) in the range of 10% to 20% dietary fat intake. However, as indicated in the last column of the table, the magnitude of tumor inhibition in CLA-treated rats was fairly consistent across all fat groups: 56% reduction in the 10% fat diet, 46% reduction in the 13.3% fat diet, 51% reduction in the 16.7% fat diet, and 50% reduction in the 20% fat diet. This observation suggests that the efficacy of CLA in mammary cancer prevention is independent of the level of fat in the diet.

The uninvolved (non-tumor bearing) mammary glands of rats from selected groups were processed for fatty acid analysis in the neutral lipid and phospholipid fractions. The results from 4 dietary treatment groups (10% and 20% fat $\pm$ CLA) are presented in Table 2. The data are expressed as percentages of total fatty acids. As indicated in footnote *b*, each value represents the mean of 7-8 samples, but since the standard error of the group mean is generally within 5% of the mean, the SEM is omitted from the table in order to make it more readable.

In the neutral lipid fraction, the three predominant fatty acids were C16:0, C18:1 and C18:2. In rats fed 10% and 20% fat without CLA, the most significant change was an increase of C18:2 incorporation ($P<0.05$) in the 20% fat group. The feeding of 1% CLA in diets containing 10% and 20% fat increased the netural lipid CLA content by 17.5-fold (from 0.2% to 3.5%) and

12-fold (from 0.2% to 2.4%), respectively, but did not alter the proportion of the other fatty acids in any substantial way.

Analysis of the phospholipid fraction showed that C16:0, C18:0, C18:2 and C20:4 accounted for >90% of total fatty acids. In particular, the high level of C20:4 incorporation was a distinctive characteristic of phospholipids. Thus the fatty acid profile found in phospholipids was different from that found in neutral lipids. There was again no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in phospholipids. Interestingly, the increase in CLA incorporation in phospholipids (~0.3%) was much smaller in magnitude compared to that observed in neutral lipids (~2-3%). These findings suggest that there might be some selectivity of CLA incorporation in different classes of lipid.

Table 3 shows the mammary cancer chemopreventive activity of CLA in rats fed either an unsaturated fat (corn oil) or a saturated fat (lard) diet. It was apparent from the data that CLA was equally effective in suppressing tumorigenesis regardless of the type of dietary fat intake. Furthermore, the magnitude of tumor inhibition seen in this experiment was very similar to that described in the first experiment (Table 1). In other words, with a constant dose of DMBA, the feeding of 1% CLA reduced the number of mammary tumors by about one-half, and this activity was evidently unaffected by the fat content (level or type) in the diet.

The efficacy of CLA in inhibiting lipid peroxidation and oxidative damage in mammary tissue was assessed by measuring malondialdehyde in mammary gland homogenate and 8-OHdG in mammary gland DNA. The results are presented in Table 4. In this experiment, rats were fed the same corn oil or lard diet, with or without 1% CLA, as in the mammary carcinogenesis

experiment shown in Table 3. However, the animals were not treated with DMBA and they were sacrificed after 2 months of feeding. Malondialdehyde levels were significantly elevated in rats fed the corn oil-versus the lard-diet ($p<0.001$), this finding thus confirms the increased susceptibility of unsaturated fat to peroxidation. The feeding of CLA was associated with a reduction of malondialdehyde in the mammary tissue in both fat groups ($p<0.001$). This effect was somewhat greater in rats fed a rich unsaturated fat diet (corn oil, 35% reduction; lard, 25% reduction, $p=0.02$). Diet-associated differences in tissue levels of 8-OHdG were less remarkable. A 10-15% increase of 8-OHdG levels was detected with feeding the corn oil- versus the lard-diet ($p=0.08$); however, tissue levels of this oxidized base were unaffected by CLA ($p=0.42$).

It has been reported previously that 1% CLA produced a maximal inhibitory effect on mammary carcinogenesis in rats fed a 5% corn oil diet (6). No further protection was detected at levels above 1% CLA. In order to find out whether the dose response to CLA might be different in rats fed a 20% corn oil diet, an experiment was carried out to evaluate such a possibility (the protocol was otherwise identical to the previous 5% corn oil experiment with CLA supplemented at 0.5%, 1% or 1.5%). As pointed out in the Methods section, the difference in linoleate intake is substantial between a 5% and a 20% corn oil diet. If the action of CLA is totally dissociated from the availability of linoleic acid, the dose response to CLA is likely to be the same in rats consuming either a 5% or 20% corn oil diet. The results in Table 5 clearly show that maximal tumor inhibition was obtained with 1% CLA in rats fed a 20% corn oil diet. Increasing the concentration of CLA to 1.5% did not lead to a greater benefit in cancer protection.

Discussion

CLA is not the only fatty acid known to inhibit carcinogenesis. Eicosapentaenoic acid and docosahexaenoic acid, which are representative of the n-3 polyunsaturated fatty acids in fish oil, also fit this category (16). However, CLA differs from the fish oil fatty acids in two distinct aspects as far as their efficacies are concerned. Whereas fish oil is usually required at levels of about 10%, CLA at levels of 1% or less is sufficient to produce a significant cancer protective effect (7). Additionally, there are a number of papers which have indicated that an optimal ratio of fish oil to linoleate in the diet is critical in achieving maximal tumor inhibition (17-19). As can be seen from the present study, the potency of CLA in cancer prevention is largely dissociated from the quantity and type of dietary fats consumed by the host.

A possible mechanism of cancer prevention by fish oil n-3 polyunsaturated fatty acids has been postulated to be through perturbation of eicosanoid biosynthesis (19,20). In vivo, linoleic acid is converted to arachidonic acid which is the precursor for the various eicosanoids produced via either the cyclooxygenase or lipoxygenase pathway. The data presented in this paper tend to suggest that CLA is unlikely to interfere with the metabolic cascade involved in converting linoleic acid to eicosanoids. First, the anticarcinogenic efficacy of CLA was not affected by the variations in linoleate intake as demonstrated by the experiments reported in Tables 1 and 3. Second, a similar dose response to CLA at 1% and below was noted in rats fed either a 5% or 20% corn oil diet (Ref. 6 and Table 5 of present paper). No further protection was evident with supplementation of CLA above 1% in both cases. The fact that the effect of CLA maximizes at 1% may indicate a limiting step in the capacity to metabolize CLA to some active product(s)

which is essential for inhibition of carcinogenesis. Suffice it to note that the absorption of CLA is probably not a confounding factor here because the tissue accumulation of CLA continues to rise with dietary levels above 1% (unpublished data).

In all the carcinogenesis experiments included in this paper, CLA was given to the animals starting at 1 week before DMBA and continuing until termination of the experiment. We adopted this protocol initially with the experiment shown in Table 1, and in order to maintain uniformity, followed the same protocol in subsequent experiments reported in Tables 3 and 5. However, we had recently found that CLA did not affect DMBA binding to mammary cell DNA (7), nor did it affect the phase II conjugating enzymes such as glutathione-S-transferase and UDP-glucuronyl transferase (6). In other words, CLA is expected to have little influence on DMBA activation or detoxification. It can thus be conjectured that the major impact of CLA on mammary carcinogenesis with the above protocol is due to its inhibitory effect on tumor promotion or progression.

Some explanation is called for here about the finding that in rats which were maintained on the "vegetable fat blend" diet, there was a small but detectable amount of CLA in the mammary tissue even though the animals did not receive an exogenous supply of CLA. In an attempt to determine whether the bacterial flora in the colon of rats could be the source of CLA, Chin et al (21) have recently examined the tissue levels of CLA between conventional and germ-free rats which were fed diets with or without free linoleic acid. With the conventional rats, tissue CLA concentrations were 5-10 times higher in those animals given a 5% linoleic acid supplement. In contrast, CLA concentrations in tissues of germ-free rats were not affected by the addition of

linoleic acid. These findings strongly suggest that the intestinal bacterial flora of rats is capable of converting linoleic acid to CLA.

As shown by the data in Table 2, there might be some selectivity in the incorporation of CLA into different lipids following the ingestion of a diet rich in CLA. When expressed as a percentage of total fatty acids, CLA is more abundant in neutral lipids than in phospholipids. It is unclear whether this uneven distribution of CLA in various lipid fractions may have any relevance in cancer risk modulation. Because of the configuration of the *trans* double bond(s) in CLA, the incorporation of CLA in membrane phospholipids could conceivably diminish the fluidity of the lipid bilayer. On the other hand, the small amount of CLA in phospholipids tends to argue against the significance of a membrane effect. The storage of CLA in neutral lipids could portend the importance of this pool in providing a continuous supply of CLA for the generation of some active metabolite(s). Further research is needed to examine the rate of turnover of CLA in neutral lipids and the possible oxidative modification of CLA similar to that observed with linoleic acid (22-24).

The ability of CLA to suppress lipid peroxidation was first described by Pariza's laboratory (9). In that work, linoleic acid was exposed to air and moderate heat, with or without a very small amount of CLA, for an extended period of time. Under those conditions, the degree of linoleic acid oxidation (peroxide value) was determined by the thiocyanate method (25). It was hypothesized that an oxidized derivative of CLA might be the active antioxidant species rather than CLA itself (9). According to the proposed scheme which is supported by spectrophotometric evidence, a β-hydroxy acrolein moiety would be introduced across the

conjugated double bond of CLA following reaction with a hydroxyl or peroxyl radical and molecular oxygen. Antioxidant activity would result from chelation of iron by the β-hydroxy acrolein functional group, thereby interfering with the Fenton reaction. A recent paper by van den Berg (26), however, contradicted the above conclusion. These investigators studied whether CLA could protect membrane vesicles composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under various conditions. Oxidation was determined by direct spectrophotometric measurement of conjugated diene formation and by gas chromatographic/mass spectrometric analysis of fatty acids. It was found that CLA neither acts as a radical scavenger nor is it converted into a metal chelator in the Fe^{2+}-ion dependent oxidative reaction. Thus at least in a model membrane system, CLA does not function as an effective antioxidant or antioxidant precursor.

The results presented in Table 4 may provide new clues of the effect of CLA on oxidative events *in vivo*. Malondialdehyde levels were lower in mammary tissue of CLA-treated rats, and the suppressive effect was somewhat greater in rats fed the more unsaturated dietary fat. Since malondialdehyde was measured in whole mammary gland homogenate, it is likely to represent the peroxidation of neutral lipids which are found predominantly in the mammary gland adipocytes. As shown in Table 2, CLA is also preferentially incorporated in the neutral lipid fraction. On the other hand, the levels of 8-OHdG, which are only marginally affected by the type of dietary fat and not at all by CLA supplementation, are probably a better indicator of DNA oxidative damage that may be causally related to tumor promotion/progression. The presence of 8-OHdG has been implicated in mismatching errors and base substitutions in DNA replication (27,28). The absence

of a detectable effect of CLA on 8-OH-dG is also consistent with the lack of a significant accumulation of CLA in the phospholipid fraction which is likely to originate from the mammary epithelial cells. In summary, based on the information obtained in this study, we believe that the ability of CLA to inhibit mammary carcinogenesis is not mediated by protecting the target cell DNA against damage induced by reactive oxygen species. Current research is focused on using a mammary epithelial cell culture model (29,30) to generate new insights of potential mechanisms of CLA in regulating growth and differentiation.

Acknowledgments

The authors thank Todd Parsons and Cathy Russin for technical assistance with the experiments. This work was supported by Grant No. CA 61763 from the National Cancer Institute, National Institutes of Health, to Clement Ip, Grant No. AIBS 2423 from the Department of Defense to Henry Thompson, and a gift from Kraft Foods, Inc., Glenview, IL.

References

1. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989) Newly recognized anticarcinogenic fatty acids: Identification and quantification in natural and processed cheeses. *J. Agricul. Food Chem.*, **37**, 75-81.

2. Chin, S.F., Liu, W., Storkson, J.M., Ha, Y.L., and Pariza, M.W. (1992) Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *J. Food Comp. Anal.*, **5**, 185-197.

3. Ip, C., Carter, C.A., and Ip, M.M. (1985) Requirement of essential fatty acid for mammary tumorigenesis in the rat. *Cancer Res.*, **45**, 1997-2001.

4. Fischer, S.M., Conti, C.J., Locniskar, M., Belury, M.A., Maldve, R.E., Lee, M.L, Leyton, J., Slaga, T.J., and Bechtel, D.H. (1992) The effect of dietary fat on the rapid development of mammary tumors induced by 7,12-dimethylbenz(a)anthracene in SENCAR mice. *Cancer Res.*, **52**, 662-666.

5. Welsch, C.W. (1992) Relationship between dietary fat and experimental mammary tumorigenesis. A review and critique. *Cancer Res.*, **52**, 2040s-2048s.

6. Ip, C., Chin, S.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Res.*, **51**, 6118-6124.

7. Ip, C., Singh, M., Thompson, H.J., and Scimeca, J. (1994) Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Res.*, **54**, 1212-1215.

8. Ip, C., Scimeca, J.A., and Thompson, H. (1995) Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. *Nutr. Cancer*, **24**, 241-247.

9. Ha, Y.L., Storkson, J., and Pariza, M.W. (1990) Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.*, **50**, 1097-1101.

10. Park, J.-W. and Floyd, R.A. (1992) Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Radic. Biol. Med.* **12**, 245-250.

11. Haegele, A.D., Briggs, S.P., and Thompson, H.J. (1994) Antioxidant status and dietary lipid unsaturation modulate oxidative DNA damage. *Free Rad. Biol. Med.*, **16**, 111-115.

12. Horvath, P.M., and Ip, C.. (1983) Synergistic effect of vitamin E and selenium in the chemoprevention of mammary carcinogenesis in rats. *Cancer Res.*, **43**, 5335-5341.

13. Ip, C. (1990) Quantitative assessment of fat and calorie as risk factors in mammary carcinogenesis in an experimental model. *Prog. Clin. Biol. Res.*, **346**, 107-117.

14. Ip, C. (1987) Fat and essential fatty acid in mammary carcinogenesis. *Am. J. Clin. Nutr.*, **45**, 218-224.

15. Draper, H.H. and Hadley, M. (1990) Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology* **186**,421-431.

16. Cave, W.T., Jr. (1991) Dietary n-3 polyunsaturated fatty acid effects on animal tumorigenesis. *FASEB J.*, **5**, 2160-2166.

17. Ip, C., Ip, M.M., and Sylvester, P. (1986) Relevance of trans fatty acids and fish oil in animal tumorigenesis studies. *Prog. Clin. Biol. Res.*, **222**, 283-294.

18. Cohen, L.A., Chen-Backlund, J.-Y, Sepkovic, D.W., and Sugie, S. (1993) Effect of varying proportions of dietary menhaden and corn oil on experimental rat mammary tumor promotion. *Lipids*, **28**, 449-456.

19. Rose, D.P., Rayburn, J., Hatala, M.A., and Connolly, J.M. (1994) Effects of dietary fish oil on fatty acids and eicosanoids in metastasizing human breast cancer cells, *Nutr. Cancer,* **22**, 131-141.

20. Rose, D.P. and Connolly, J.M. (1993) Effects of dietary omega-3 fatty acids on human breast cancer growth and metastasis in nude mice. *J. Nat. Cancer Inst.*, **85**, 1743-1747.

21. Chin, S.F., Storkson, J.M., Liu, W., Albright, K.J., and Pariza, M.W. (1994) Conjugated linoleic acid (9,11- and 10,12-octadecadienoic acid) is produced in conventional but not germ-free rats fed linoleic acid. *J. Nutr.*, **124**, 694-701.

22. Buchanan, M.R., Haas, T.A., Legarde, M., and Guichardant, M. (1985) 13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J. Biol. Chem.*, **260**, 16056-16059.

23. Bull, A.W., Nigro, N.D., and Marnett, L.J. (1988) Structural requirements for stimulation of colonic cell proliferation by oxidized fatty acids. *Cancer Res.*, **48**, 1771-1776.

24. Baer, A.N., Costello, P.B., and Green, F.A. (1990) Free and esterified 13(R,S)-hydroxyoctadecadienoic acids: principal oxygenase products in psoriatic skin scales. *J. Lipid Res.*, **31**, 125-130.

25. Ramarathnam, N., Osawa, T., Namiki, M., and Kawakishi, S. (1988) Chemical studies on novel rice hull antioxidants. 1. Isolation, fractionation, and partial characterization. *J. Agric. Food Chem.*, **36**, 732-737.

26. van den Berg, J.J.M., Cook, N.E., and Tribble, D.L. (1995) Reinvestigation of the antioxidant properties of conjugated linoleic acid. *Lipids,* **30,** 599-605.

27. Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., and Nishimura, S. (1987) Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. *Nature,* **327**, 77-79.

28. Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. *J. Biol. Chem.,* **267**, 167-172.

29. Darcy, K.M., Shoemaker, S.F., Lee, P.-P. H., Vaughan, M.M., Black, J.D., and Ip, M.M. (1995) Prolactin and epidermal growth factor regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture. *J. Cell Physiol.,* **163**, 346-364.

30. Darcy, K.M., Shoemaker, S.F., Lee, P.-P. H., Ganis, B.A. and Ip, M.M. (1995) Hydrocortisone and progesterone regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture. *J. Cell Physiol.,* **163**, 365-379.

Table 1. Mammary cancer prevention by CLA in rats fed different levels of fat[a]

Dietary fat level	CLA	Tumor incidence	Total No. of tumors	% inhibition[b]
10%	----	68.8%	71	
10%	1%	40.6%[c]	31[c]	56%
13.3%	----	81.3%	74	
13.3%	1%	46.9%[c]	40[c]	46%
16.7%	----	87.5%	94	
16.7%	1%	59.4%[c]	46[c]	51%
20%	----	90.6%	98	
20%	1%	59.4%[c]	49[c]	50%

[a] The fat used in this experiment was the "vegetable fat blend" as described in the Methods section. There were 32 rats per group.

[b] Percent inhibition was calculated using the tumor number data.

[c] $P<0.05$ compared to the corresponding control group without CLA.

Table 2. CLA incorporation in neutral lipid and phospholipid fractions of mammary gland[a]

Fatty acid	Neutral lipid[b] 10% fat -CLA	Neutral lipid[b] 10% fat +CLA	Neutral lipid[b] 20% fat -CLA	Neutral lipid[b] 20% fat +CLA	Phospholipid[b] 10% fat -CLA	Phospholipid[b] 10% fat +CLA	Phospholipid[b] 20% fat -CLA	Phospholipid[b] 20% fat +CLA
C12:0	1.2	1.2	1.6	1.6				
C14:0	1.7	1.9	1.7	1.6	1.1	1.1	1.2	1.1
C16:0	24.3	24.7	21.3	20.5	19.6	19.5	20.4	21.1
C16:1	3.9	3.9	3.0	1.5	0.5	0.4	0.4	0.3
C18:0	3.9	3.6	3.7	4.8	37.3	38.1	38.2	37.6
C18:1	42.3	38.9	40.4	38.8	5.1	4.9	4.6	4.8
C18:2	20.9	21.0	26.2	27.0	11.5	11.3	10.7	11.6
C18:3	0.9	0.9	1.3	1.2	0.5	0.4	0.4	0.3
C20:4	0.7	0.4	0.6	0.6	24.3	23.9	23.3	23.2
CLA	0.2	3.5	0.2	2.4	0.1	0.4	0.1	0.4
	100	100	100	100	100	100	100	100

[a] The samples were processed from uninvolved glands of rats reported in Table 1.

[b] Results are expressed as % of total fatty acids. The sum of each column is equal to 100%. Each value represents the mean of 7-8 samples, the SEM is generally within 5% of the mean.

Table 3. Mammary cancer prevention by CLA in rats fed either an unsaturated fat or a saturated fat diet[a]

Dietary fat	CLA	Tumor incidence	Total No. of tumors	% inhibition[b]
corn oil	—	83.3%	68	
corn oil	1%	40.0%[c]	35[c]	49%
lard	—	80.0%	60	
lard	1%	40.0%[c]	32[c]	47%

[a] The unsaturated fat diet contained 20% corn oil, while the saturated fat diet contained 8% corn oil +12% lard. There were 30 rats per group.

[b] Percent inhibition was calculated using the tumor number data.

[c] $P<0.05$ compared to the corresponding control group without CLA.

Table 4. Effect of CLA feeding on malondialdehyde and 8-OHdG levels in mammary gland[a,b]

Dietary fat	Malondialdehyde (nmol/mg protein)[c] -CLA	Malondialdehyde (nmol/mg protein)[c] +CLA	8-OHdG (residues/10^6 dG)[d] -CLA	8-OHdG (residues/10^6 dG)[d] +CLA
corn oil	1.39±0.08	0.90±0.14	4.00±0.26	4.05±0.20
lard	0.43±0.03	0.32±0.02	3.38±0.26	3.75±0.30

[a] Rats were fed either the corn oil or lard diet, with or without 1% CLA, for 2 months.

[b] Results are expressed as mean ± SE (n=9).

[c] By factorial analyses of variance, the following effects on malondialdehyde were noted. Type of fat:F-ratio 88.903, $p<0.001$; CLA:F-ratio 13.76, $p=0.001$; Interaction between fat type and CLA:F-ratio 5.62, $p=0.024$.

[d] By factorial analyses of variance, the following effects on 8-OH-dG were noted. Type of fat:F-ratio 3.18, $p=0.08$; CLA:F-ratio 0.42, $p=0.42$; Interaction between fat type and CLA:F-ratio 0.37, $p=0.54$.

Table 5. Mammary cancer prevention by different levels of CLA in rats fed a 20% corn oil diet[a]

CLA	Tumor incidence	Total No. of tumors	% inhibition[b]
—	93.3%	87	
0.5%	70.0%	53[c]	39%
1%	50.0%[c]	37[c]	57%
1.5%	46.7%[c]	34[c]	61%

[a] There were 30 rats per group.
[b] Percent inhibition was calculated using the tumor number data.
[c] $P<0.05$ compared to the control group without CLA.